Analysis of the Barley Grain Protease Spectrum

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Abstract

Malting is the controlled germination of barley grains, under specific conditions and up to the point of grain modification, and is the keystone of the brewing and distilling industries. Protease enzyme activity is an important constituent of this process as not only does this catalyse the degradation of the grains protein stores, but it also has involvement in other aspects of the germination process such as the activation of β – amylase. However, despite their importance, little is known of the identities and specific roles of the protease classes in the malting and germination process. With this in mind a biochemical and proteomic approach was taken in order to attempt to identify protease enzymes from malted and germinating barley grains and also to identify the roles of specific protease classes in barley grain malting and germination.

FPLC based protein fractionation, SDS – PAGE analysis and MALDI – ToF mass spectroscopy were used in an attempt to purify metallo and serine class proteases from extracts of four day micro malted malt and germinating barley grains. A combination of class specific protease inhibitors, germination studies, enzyme assays of both barley grain proteases and specific starch degrading enzymes, and western blotting were employed to investigate the roles of the different protease classes in both overall grain physiology during germination and specifically, in the regulation of enzymes involved in starch breakdown.

These investigations show that the serine and aspartate class proteases have a role in the positive regulation of the amounts of α – amylase present in barley grains during germination in a process that may involve gibberellic acid signalling. Furthermore, it was also shown that the serine class proteases have a role in the negative regulation of β – amylase activity during germination, thus revealing the complexity of the regulatory roles of barley grain proteases during grain germination. In addition, germination studies carried out in the presence of both the divalent cation chelator 1, 10 phenanthroline and different divalent cations revealed the potential importance of transition metal ions such as zinc, iron and manganese in the onset of grain germination.

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Declaration

I, Angela Bell, hereby declare that I am the author of this thesis. All work described in this thesis is my own, except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of references.

Angela Bell

Table of Contents

List of Figures

List of Tables

List of Abbreviations

^{o}C	Degrees Celsius
µg	Microgramme
µl	Microlitre
µm	Micrometre
µM	Micromolar
(V / V)	Volume by volume
(W / V)	Weight by volume
ABA	Abscisic Acid
ACN	Acetonitrile
BSA	Bovine serum albumin
DTT	Dithiothreitol
E – 64	*Trans* – epoxysuccinyl – L – leucylamido – 4 – guanidino) butane
EPA	Endoprotease A
EPB	Endoprotease B
FPLC	Fast Protein Liquid Chromatography
GA	Gibberellic Acid
g	Grammes
xg	times gravity (for use with centrifuge speeds)
HCl	Hydrochloric acid
KDa	Kilodalton
kg	kilogramme
Kv	Kilovolts

l	Litre
M	Molar
MALDI-TOF	Matrix Assisted Laser Desorption / Ionisation Time of Flight
mg	Milligramme
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MPa	Megapascal
MW	Molecular weight
m/z	Mass to charge ratio
NaCl	Sodium Chloride
nm	Nanometre
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
rpm	Repeats per minute
SDS	Sodium dedecyl sulphate
TCA	Trichloroacetic acid
TEMED	N, N, N', N' – tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	2 – amino – 2 – hydroxymethylpropan – 1, 3 – diol
UV	Ultraviolet
V	Volts

1: Barley

Barley (*Hordeum vulgare. L*) is a monocotyledonous cereal belonging to the genus *Hordeum*, the tribe Triticeae (Briggs, 1978) and is a member of the grass, *Gramineae*, family. In terms of global production, barley ranks as the fourth major cereal crop (after wheat, maize and rice) with over 133.85 million tonnes being produced annually worldwide (USDA, 2008). Of this global production, more than three quarters is used for animal feed with the remainder being used mainly by the brewing and distilling industries (Morris & Bryce, 2000). As well as its significant economic importance, barley is of considerable nutritional significance to the human diet contributing a significant portion of the global human diet (Finnie & Svensson, 2009) by way of both direct consumption, in the form of beer or bread for example, and indirect consumption, in the form of animals fed on barley feed. This global reliance on barley is primarily due to its ability to adapt to a wide range of environments leading to its cultivation in diverse areas such as the sub – arctic, cooler tropical highlands and the sub – tropics, temperate regions, high altitudes and the saline soils of maritime area. Indeed, one of the few places on Earth that barley is not found is the more hot and humid regions such as the tropical lowlands.

1.1: Cultivated Barley – Its Origins and Main Varietal Classification

There are many different varieties of barley grown around the world today, all of which can be separated in two main groups depending upon the number of rows of grain that can be observed when looking at an ear of barley from above. These groups are referred to as the two – rowed and six – rowed barleys (Briggs, 1978). Evidence suggests that the modern day barley arose from a common two – rowed ancestor in the “fertile crescent” of the Middle East about 8000 years ago to form the progenitors of today’s barley (Briggs, 1978).

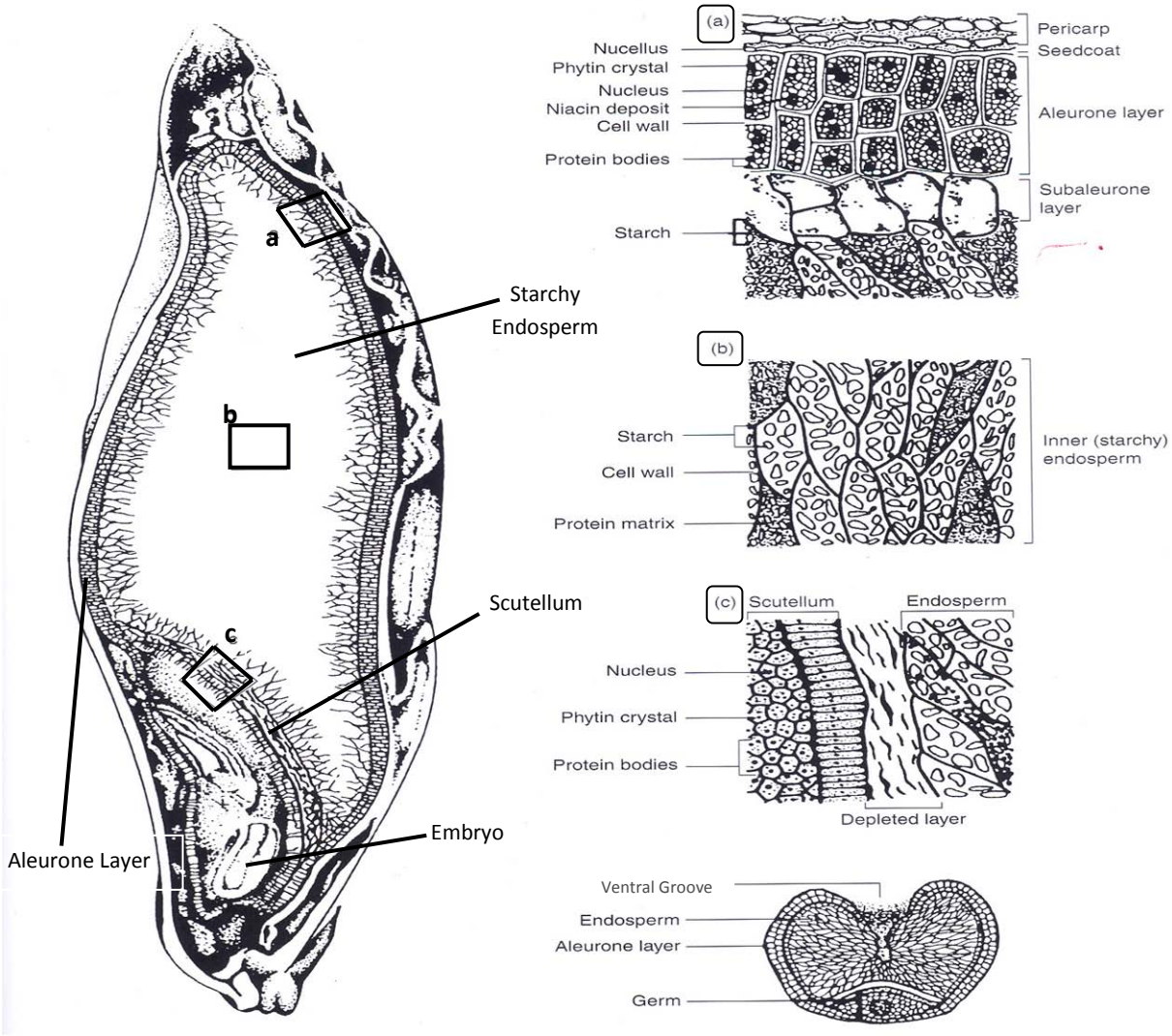
1.2: Anatomy of a Barley Grain

A mature barley grain is oval in shape, tapering at each end (Fig. 1) and has a furrow or crease running the full length of its ventral axis (Briggs, 1978). While the majority of barley grains have a protective outer husk surrounding the grain, huskless varieties can also be found but are not as widely cultivated for malting due to the inability of huskless malt to form

a natural filtration bed (as they lack the husks to do so) during wort (liquid containing carbohydrates, small peptides and amino acids that the yeast uses as a source of nutrition during fermentation) separation (Briggs, 1978).

The barley grain itself consists of five distinct tissues (Fig. 1.1); two living and three dead. In mature grains the living tissues comprise of the embryo or germ and the aleurone layer, while the endosperm, pericarp and testa (or seed coat) composes the dead tissue (Palmer, 1991). The pericarp (the wall of the grain) can be found just beneath the husk and on top of the testa. Both of these structures cover the entire length of the grain up to the awn (Fig. 1.1) and function not only as extra layers of protection for the seed, but also as semi permeable membranes allowing the passage of some substances such as water into the grain, but restricting the flow of others, such as hormones, out of the grain.

FIGURE 1.1: Structure of the Barley Grain (Adapted from Izydorczyk & Dexter, 2004)



1.2.1: The Embryo

The embryo accounts for approximately four percent of the total dry weight of the mature grain (Finnie & Svensson, 2009) and is located at its proximal end. Two main sub – organs compose the embryo, these are the axis and the scutellum. The axis consists of the root, shoot and stem precursors, whereas the scutellum functions to aid in the absorption of amino acid and other nutrients, such as glucose, from the endosperm during germination.

1.2.2: The Starchy Endosperm

This is the largest single tissue in the entire grain and composes about 87 % of the total grain dry weight (Finnie & Svensson, 2009). As the name suggests the starchy endosperm consists of starch granules, which are embedded in a protein matrix. The starch granules are present in two forms, the large or A type granules, which are biconvex in shape and range from 10 – 25 µm in size, and the small, B type granules which are spherical and 2 – 5 µm (Tester et al, 2004). The starchy endosperm also contains β – glucan (a cell wall polysaccharide which acts as further store of glucose) and storage proteins, which are found in protein bodies and comprise the endosperm's protein matrix. Differences in the distribution patterns of starch and storage proteins within the endosperm give rise to the terms mealy grain and steely grain (Chandra et al, 1999). In mealy grains there is a lower concentration of storage proteins in the endosperm, and as a consequence the protein bodies are loosely grouped, with air gaps in between them. These grains tend to be softer than the steely grains due to their less dense endosperms, and mealy grains also have higher starch levels. Steely grains on the other hand, are much harder as they possess larger areas of densely packed protein matrix (and thus less starch than the mealy grains) with no air gaps in between. This distribution of granule type and cell packing has a large impact on modification as mealy grains will modify at a quicker rate than the steely grains due to faster water, enzyme movement through their endosperm. This difference in modification pattern can be problematic during malting as uneven grain modification produces heterogeneous malt which can result in a number of brewing problems such as haze and slow beer filtration.

1.2.3: The Endosperm: A Key Site in Grain Germination

Germination requires the breakdown of starch granules, cell walls and storage proteins in order to liberate the nutrients required for the growth and development of the embryo. Germination is the opposite of its preceding stage, grain filling (Dominguez & Cejudo, 1996). These two stages can be viewed as opposing as during filling the grain accumulates storage proteins and starch granules in the endosperm which are in turn, broken down during germination to provide the embryo with the nitrogen and carbon required for seedling growth. It is the process of endosperm modification that is exploited during malting to produce grains which are modified up to a specific point (that of the production of hydrolytic enzymes and

the beginnings of endosperm degradation). One of the first stages of endosperm modification is the breakdown of the protein matrix, which houses the starch granules. This is one of the most important processes of grain germination (Jones, 2005) as it not only allows the starch degrading enzymes access to the starch granules, but also enables the breakdown of major storage proteins, such as the hordeins, glutelins, albumins and globulins, located within the protein matrix. These proteins contribute approximately 40 %, 30 %, 20 % and 10 % respectively of the total stored protein content of the grain (Shewry & Halford, 2002) and their breakdown is essential for embryonic growth and development as the embryo can only assimilate small molecules such as amino acids rather than whole proteins. Degradation of the protein matrix is achieved via the actions of proteolytic enzymes produced in the embryo and aleurone layer in response to hormonal signalling (section 1.2.5). During grain filling, when the grain is depositing its stores in the endosperm, the levels of the dormancy promoting plant hormone ABA are high thus keeping the grain in a state of dormancy. During this time the levels of the antagonistic hormone, gibberellic acid are very low. At the onset of germination the embryo begins to release gibberellic acid into the aleurone layer and ABA production is halted (Dominguez & Cejudo, 1996). Once GA has migrated into the aleurone layer it induces the production and release of hydrolysing enzymes into the endosperm thereby promoting embryonic growth and development.

1.2.4: The Aleurone Layer

Surrounding the embryo (up to the scutellum) and endosperm, the aleurone layer (Fig. 1.1) contributes approximately nine percent of the total grain dry weight (Finnie & Svensson, 2009). Unlike the embryo, which is diploid, the aleurone layer (and the endosperm) is triploid (Palmer, 1992). During germination the aleurone layer performs a major role as it is the grains major site of hydrolase enzyme synthesis and release.

During grain development the aleurone layer (along with the starchy endosperm) is formed during a series of highly organised divisions of the cells surrounding the large central vacuole of the undeveloped endosperm (Richie et al, 2000). This process continues until this large vacuole has been completely filled with new cells ready to differentiate into the aleurone layer and starchy endosperm. Of the cells comprising the aleurone layer, and in

addition to the cells that secrete hydrolases in response to gibberellic acid (see section 1.2.5), there are three unique cell types that can be found at specific regions around the grain (Richie et al, 2000). The first is located at the grain's ventral groove. These cells function during endosperm development as a site of nutrient transfer, and, during grain germination, as a site of nutrient storage and hydrolase production (Richie et al, 2000). The second cell type encircles the outer edges of the embryo. Due to their location away from the endosperm these cells are not thought to be involved in the production of hydrolases but are instead thought to have a role in controlling the transit of water into and out of the embryo, and to also function as protectors of the embryo, guarding against pathogen invasion (Richie et al, 2000). The final class of cells are located in direct contact with the starchy endosperm and are referred to as the sub - aleurone cells. These cells are very similar to those of the endosperm as they too die upon grain maturation, and house storage proteins and starch granules. Since these cells are not viable in the mature grain, they are thought to have no role in the production of hydrolytic enzymes.

The aleurone's ability to produce and release hydrolytic enzymes during germination is subject to tight hormonal regulation (Dominguez & Cejudo, 1996) involving the antagonistic actions of the plant hormones GA (gibberellic acid – produced during germination to promote nutrient mobilisation) and ABA (abscisic acid – produced during grain dormancy).

1.2.5: Germination, Gibberellic Acid & the Aleurone Layer

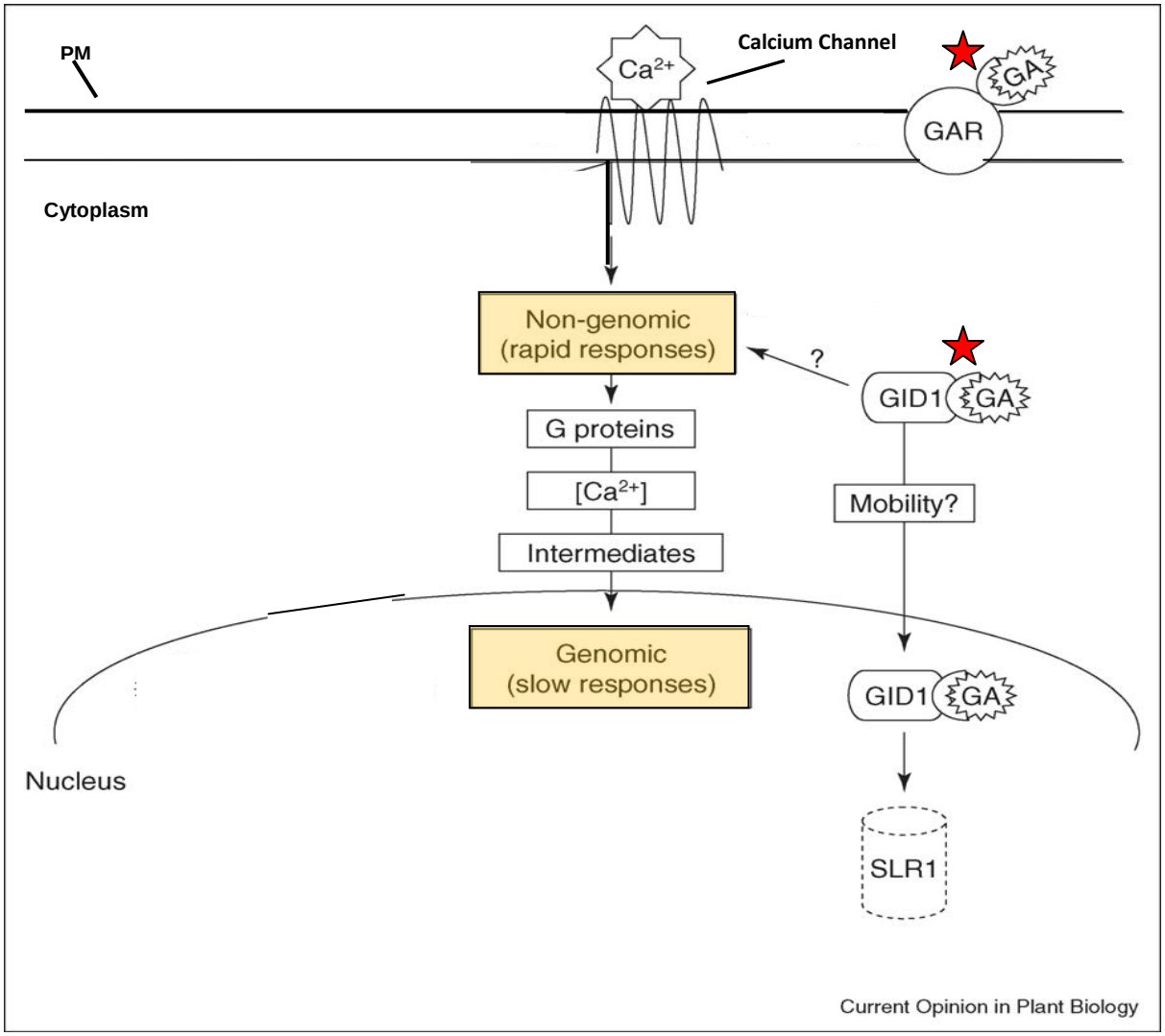
Grain germination is initiated by the uptake of water by the dry, dormant grain (imbibition) and is completed by the extension of the radical (embryonic root) out through the husk of the grain (chitting) (Bewley, 1997). The function of germination is to mobilise the grains stores of nutrients (in a process known as modification) and to harness them in the growth and development of the embryonic plant. Modification is thus an essential component of the germination process, and involves the actions of, amongst other things; reducing agents, plant hormones such as the gibberellins, and hydrolytic enzymes in the complex and often interlinking web of biochemical processes that play their part in this process of nutrient mobilisation and plant development.

As previously mentioned, imbibition marks the beginning of grain germination. This process brings with it a change in hormonal signalling from that of ABA (promoting grain dormancy) to that of GA (promoting grain germination and seedling growth) (Dominguez & Cejudo, 1996). In resting mature grains ABA functions to repress metabolic activity thus maintaining the grain in its dormant state. As the moisture content of the grain increases during imbibition ABA production ceases and is replaced by that of other plant hormones such as ethylene and GA. Ethylene is a gaseous hormone produced in most plant tissues (Locke et al, 2000) and has diverse roles in many developmental processes including germination (Linkies & Leubner – Metzger, 2011). During germination, ethylene functions, through processes which are not fully understood but probably involving the antagonism of ABA signalling (Linkies & Leubner - Metzger, 2011), to break grain dormancy and promote germination. A lot more is known, however, about gibberellic acid. Gibberellic acid is produced by the embryo and promotes grain germination and embryonic growth by the induction of genes encoding the production of hydrolytic enzymes such as proteases, α – amylase and β – glucanase by the aleurone layer (Peng & Harberd, 2002). These enzymes are then released into the endosperm where modification takes place (O'Brien et al, 2010). The entire modification process takes approximately five days from the initial production of GA by the embryo, its subsequent diffusion through the scutellum and into the aleurone layer, the triggering of hydrolase production, their subsequent diffusion into the endosperm, and the induction of endospermal modification. This modification process manifests as a well defined planar front of enzymatic activity (O'Brien et al, 2010) originating close to the scutellum / aleurone junction, where the production and release of hydrolases begins, and propagates along the entire length of the grain in a direction away from the scutellum.

The mechanisms of GA perception, signal transduction and subsequent gene expression by the aleurone involve a complex of signalling pathways, second messenger systems and transcriptional regulators (Chebotar & Chebotar, 2011). Until recently evidence suggested that the GA receptor was located on the plasma membrane of aleurone cells and that, unlike the ABA receptors, there were no cytoplasmic GA receptors present in aleurone cells. This evidence came from studies where GA was microinjected into isolated aleurone protoplasts and no stimulation of α – amylase gene expression (α – amylase genes are switched on by GA signalling) was observed. However, when GA was included in the protoplast incubation media the expression of α – amylase genes occurred (Lovegrove & Hooley, 2000). This

coupled with the observation that GA_4 covalently bound to Sepharose beads (and thus membrane impermeable) stimulated high levels of α – amylase activity lead to the hypothesis that the GA receptor was plasma membrane bound and not internal in the aleurone layer cells. However, a loss of function mutant termed *gibberellin insensitive dwarf 1* (*gid1*) has recently been analysed in rice (Ueguchi –Tanaka et al, 2005). The *gid1* gene codes for a novel nuclear membrane bound (and thus internal) GA receptor, GID1. When GID1 binds biologically active GA the receptor interacts with the DELLA protein SLR1 (slender 1) (DELLA proteins are negative regulators of GA signalling) promoting its ubiquitination and subsequent degradation via the 26S proteasome pathway, thus relieving the DELLA mediated inhibition of GA induced genes. Rice plants with the *gid1* loss of function mutation display GA – insensitive phenotypes, such as severe dwarfism, which cannot be reversed by subsequent GA treatment. This localisation of the GID1 receptor to the nuclear membrane is not the only aspect of its functioning to make it unusual (Razem et al, 2006) as unlike many other plant hormone receptors GID1 has a very short signalling cascade which is wholly independent of any relay intermediates and relies only on protein – protein interactions in a GA – dependent manner. What's more, the interactions of the GID1 receptor with SLR1 are entirely independent of any protein kinase processes relying instead on ubiquitination of the SLR1 protein and not phosphorylation as many other plant hormone – protein interactions do. Thus the discovery of GID1 has added a further layer of complexity in the process of understanding GA signalling as the classical model of a single plasma membrane bound GA receptor inducing a branched signalling pathway activating both the genomic and non – genomic GA responses has now to give way to a revised hypothesis inclusive of the existence of this internal GA receptor. One such hypothesis is that a parallel pathway could exist (Fig. 1.2) with separate receptors for the genomic and non – genomic GA responses (Razem et al, 2006), and that there could also be tissue and maybe even compartment – specific GA receptors which trigger compartment – specific responses.

FIGURE 1.2: GA and ABA Signal Transduction in the Aleurone Layer (Adapted from Razem et al, 2006)



Where: ★ indicates GA receptors, PM = plasma membrane, GAR = membrane bound GA receptor, GID1 = nuclear GA Receptor. It is thought that the GAR receptor is responsible for the non – genomic responses to GA including changes in cytoplasmic Ca^{2+} levels and the changes to other intermediates in the signalling pathway/s including G – proteins. GID1 on the other hand is thought to activate the genomic responses to GA, and the non – genomic response of changing protein shuttling between the cytoplasm and the nucleus enabling the termination of SLR1.

The non – genomic responses to GA signalling are thought to be triggered by GA binding to the plasma membrane bound GAR receptor (Fig. 1.2) (Razem et al, 2006). These early events include an increase in the levels of cytoplasmic Ca^{2+} (Lovegrove & Hooley, 2000). This increase is thought to be brought about by a (possibly heterotrimeric G protein mediated) GA induced change in the Ca^{2+} flux though one or more plasma membrane bound

Ca^{2+} channels. This increase in intracellular calcium is accompanied by an increase in intracellular levels of calmodulin (CaM) indicating a role for the Ca^{2+} / CaM signalling pathway from the early stages of GA signal transduction. The possible targets of this signalling pathway within the aleurone could include aspects of cellular metabolism (such as the production of a CaM – activated Ca^{2+} - ATPase located on the endoplasmic reticulum) involved in the production and release of hydrolase enzymes. The CaM – activated Ca^{2+} - ATPase has been implicated in supporting the endoplasmic reticulum's increased requirement for Ca^{2+} brought about by the secretion of Ca^{2+} dependent proteins such as α – amylase (Lovegrove & Hooley, 2000). In studies involving the treatment of isolated aleurone cells, the increase in intracellular Ca^{2+} levels occurs quickly after GA treatment (after only 2 to 5 minutes in isolated wheat aleurone cells) and the synthesis and secretion of hydrolytic enzymes occurs several hours later (Lovegrove & Hooley, 2000), thus it has been proposed that this Ca^{2+} / CaM signalling pathway may be an important intermediary in the GA signalling process as if its only role lay only in the supply of Ca^{2+} for the secretion of Ca^{2+} dependent proteins, then there would be no requirement for the early increase in Ca^{2+} and CaM levels as the calcium dependent proteins are not synthesised until several hours after the initial increase in cytoplasmic calcium levels.

As previously mentioned, the genomic responses to GA signalling are thought to be initiated when GA binds to and thus activates the GID1 receptor (Murase et al, 2008). Once activated GID1 associates with DELLA proteins though a mechanism that results in the ubiquitination and destruction of the DELLA proteins by the 26S proteasome complex. This ubiquitination process is catalysed by the recruitment of GID1 associated DELLAs by the ubiquitin ligase E3 SKP1 – CULLIN – F – box (SCF) complex. This complex tags the DELLAs with ubiquitin thus targeting them for destruction by the 26S proteasome complex (Murase et al, 2008). DELLA destruction enables the transcription of GA – responsive genes such as those coding for proteases, α – amylase (section 1.8.2) and β – glucanases.

1.3: Hormones, Redox Agents and Barley Grain Germination

It is not just changes in hormonal signalling that are important for germination and grain modification, but also changes in the biochemical environment within the grain, including

changes in its oxidation – reduction state. The redox state of a biological system is very important to its proper functioning as many of the regulatory proteins and enzymes active within it are sensitive to changes in the system's redox state (Marx et al, 2002). One of the key players in the arena of cellular redox regulation is the thioredoxin protein family (Gelhaye et al, 2005). These small proteins are found ubiquitously in all organisms and are characterised by the presence of a conserved dicysteine active site (i.e. there are two highly conserved cysteine residues in the active site that are necessary for catalysis) that catalyses the thiol – disulphide interchange between cysteinyl residues on a host of target proteins resulting in their activation, inactivation or a change in their susceptibility to proteolytic breakdown. In plants there are two known thioredoxin systems, one involved in the regulation of photosynthesis and located in the chloroplasts (the *f* and *m* thioredoxins), and the other (the *h* thioredoxins) located mainly in the cytoplasm, and to a lesser extent in the mitochondria and endoplasmic reticulum, which catalyses the reduction of disulphide bonds via the NADPH – dependent thioredoxin reductase (NTR) / thioredoxin system (Shahpiri et al, 2008). The thioredoxin *h* system is very important during both grain germination and maturation with roles in the regulation of grain dormancy, storage protein mobilisation, translational control and protection against reactive oxygen species (Marx et al, 2003). At the onset of grain germination changes occur in the redox state of the aleurone layer, scutellum, endosperm and the embryo, with the embryo undergoing reduction at a faster rate, peaking at day one post imbibition as opposed to day two for the endosperm (Marx et al, 2003). Since thioredoxins are plastidic proteins and starch is laid down in the developing endosperm in the plastids (Emes et al, 2003), thioredoxin *h* should therefore be already present in the germinating endosperm. However, it has been described that the endosperm undergoes reduction later than the other parts of the grain, and the levels of thioredoxin *h* transcripts are known to increase in the aleurone layer and scutellum during wheat grain germination (Serrato et al, 2001). This, together with the aleurone layer, in response to GA signalling, undergoing cell wall degradation during early germination (12 hours after GA exposure) and full apoptosis after five days of germination (Fath et al, 2000), could mean that the later onset of reducing conditions in the endosperm is a consequence of the requirement of the endosperm for *de novo* synthesised thioredoxin *h* from the aleurone layer (released via aleuronal cell wall degradation and apoptosis) for reduction to occur. This hypothesis is supported by the observation that the redox changes occurring within the grain are concurrent with the onset of GA signalling at the aleurone membrane indicating that thioredoxins and the GA signalling pathways could operate collectively during grain

modification (Marx et al, 2003), and that thioredoxins may enter the endosperm via GA mediated aleuronal cell wall degradation and cell apoptosis. The interplay between thioredoxins and GA signalling is further supported by evidence showing that in the endosperm, thioredoxin *h* proteins reduce the previously oxidised storage proteins (such as hordeins) thereby making them digestible by proteolytic enzymes (Joudreir et al, 2005). Moreover, particular enzymes involved in grain modification such as α – amylase, limit dextrinase and trypsin like proteases are subject to inactivation by inhibitor proteins prior to the onset of germination. This inactivation is relieved in part, by the actions of thioredoxin *h* proteins which reduce the disulphide bonds between the inhibitor and the enzyme thereby making the inhibitor more susceptible to proteolytic degradation, and activating the enzyme (Kobrehel et al, 1991).

1.4: Malting

Malting is the controlled germination of barley grains, under specific conditions and to a specific point in the germination process (malting is stopped when the grain is said to be well modified) and is the keystone of the brewing and distilling industries (Jones, 2005). During the malting process barley grains undergo a series of steeping and air resting phases followed by germination, in order to germinate them up to the point at which the production of hydrolase enzymes is complete. During the steeping phase dry grain are immersed in large vats of water to bring about an increase in the grain's internal moisture content from around 10 – 12 % to 42 – 46 % (Lewis, 2001). This process is extremely important to malt quality as the grain is required to reach a specific internal moisture content (42 – 46 %) for homogeneous modification, if this moisture content is not reached then the grains could either remain unmodified or modification could take place unhomogeneously resulting in poor quality malt and a low grade finished product. Once the barley grains have reached their required germination stage and thus are appropriately modified (usually after three to five days of malting depending upon malting conditions) the grains can be used directly as "green malt" or they can undergo kilning so the malt can be stored. Green malt cannot be stored as it is still metabolically active, so as a result is usually used directly after completion of the malting process. Kilning is accomplished by the application of hot dry air to the malt which decreases the grains internal moisture levels to around five to eight percent, thus arresting germination and yielding grains that can be stored (under the correct conditions)

until further use. However, this is not the only function of the kilning process as it also plays a part in the development of malt flavour and colour compounds via the utilization of different kilning parameters ((Pyler & Thomas, 1991). For example, roasting malt up to a final kilning temperature of 230 ^{o}C will produce dark roasted malts, whereas kilning at lower temperatures up to a final kilning temperature of 75 ^{o}C to 95^{0}C will result in the production of the lighter pale ale and distilling malts (Palmer, 2006).

1.5: Protease Enzymes – Classification & Physiological Relevance

Proteases are enzymes which catalyse protein breakdown by initiating peptide bond hydrolysis. Since virtually all biological processes involve proteins, and all proteins undergo proteolysis, protease enzymes can be seen as one of the major biological regulators (Schilling & Overall, 2007). There are two major protease groups; the exoproteases and the endoproteases. Exoproteases catalysis protein hydrolysis from the terminal amino acids of their target protein molecules, in contrast to this the endoproteases act at specific amino acid residues within their substrate molecules thus breaking proteins up from the inside. Proteases can be further subdivided into one of four distinct mechanistic classes. Here, each protease class is differentiated by its mode of catalysis and its inhibition by one or more characteristic class specific inhibitors (Jones, 2005). These four mechanistic classes are;

i. Cysteine proteases. These exert their catalytic activity via a cysteine residue present in their active sites. Catalysis occurs by the formation of a covalent intermediate between the sulphur atom of the proteases cysteine residue (i.e. the nucleophile or electron donor species) and the substrate molecule's carbonyl group (i.e. the target electron – poor residue of the substrate protein molecule) (Beynon & Bond, 2001). This class of protease is specifically inhibited by *trans* – epoxysuccinyl – *L* – leucylamido – (4 – guanidino) butane (E – 64) and stimulated by reducing agents such as dithiothreitol (DTT) and β - mercaptoethanol.

ii. Metalloproteases. Metalloproteases require the presence of a metal ion (usually zinc) at their active sites and are specifically inhibited by the metal ion chelator 1, 10 phenanthroline which has a high affinity for zinc. These enzymes do not induce hydrolysis via the formation of covalent intermediates (Beynon & Bond, 2001), instead they use a mechanism whereby their metal ion is electrophilically bound to a molecule of

water thus polarising it and creating a strong nucleophilic draw to attract and hydrolyse the target protein though its carboxyl group.

iii. Aspartic proteases. These proteases, as with the metalloproteases, catalysis substrate hydrolysis without the formation of covalent intermediates. Instead, hydrolysis is brought about by the action of two active site aspartic side chains and an active site bound water molecule (again, the water molecule acts as the nucleophile) (Beynon & Bond, 2001). This group of proteases is specifically inhibited by Pepstatin A.

iv. Serine proteases. This mechanistic class is probably the most widely studied protease class in the field of enzymology (Beynon & Bond, 2001). These enzymes catalyse substrate hydrolysis via the hydroxyl group of the serine residue at their active sites in a very similar manner to that of the cysteine proteases; that is through the formation of covalent intermediates. The characteristic class specific inhibitor of serine proteases (and to a lesser extent, the cysteine proteases) is phenylmethanesulphonyl fluoride (PMSF).

Proteases have a myriad of biological responsibilities and have major roles in barley grain germination (Jones, 2005). However, despite the huge body of research dedicated to the protease enzymes as a whole, comparatively little research has been carried out into their functions in the germinating barley grain (Jones, 2005 for a review of literature in this area) and as a result the knowledge surrounding the identity and individual roles of the barley proteases and how they fit into the overall web of biochemical activity taking place during germination and malting is limited.

1.6: Barley Grain Proteases and Their Importance in Brewing and Distilling

The protease spectrum of germinating (and thus malting) barley is a complex mixture of protease classes with differing temporal and spatial expression patterns, and also differing roles in barley grain physiology (Wrobel & Jones, 1992). The levels of protease activity in mature resting barley grains is low (Jones et al, 2000); a situation which changes significantly at the onset of germination with protease activity beginning to appear at day one and reaching its maximum by approximately day three to four of the malting process

(depending upon the malting parameters). Investigations into the effects of pH and class – specific protease inhibitors on the levels of storage protein hydrolysis during barley grain germination has revealed strong evidence for the cysteine class proteases playing a key role in the degradation of storage proteins making them very important to the production of high quality malt (Jones, 2005). Furthermore, it has been estimated that during germination there could be at least 42 different proteases active within the grain (Zhang & Jones, 1995). The involvement of members of the four protease mechanistic classes was also investigated, by the addition of class specific protease inhibitors and the use of different pHs. Zhang & Jones concluded that 27 of the 42 separate activities were cysteine protease activities active at acidic pHs (pH 3.8 to 4.8), that four were aspartic protease activities, three metalloprotease activities and that eight were serine class protease activities, highlighting the strong role of the cysteine class proteases. However, since the protease activity observed during this study was identified by in – gel protease activity after electrophoresis they did not take into account post transcriptional modifications or protein breakdown products or the possible presence of Isoforms that may have resulted in the same protease being present at different locations on the gel, thus it cannot be concluded that the 42 different activity bands seen in gels were indeed 42 unique protease enzymes, but it is the only study which has attempted to provide a global overview of the number and class of proteases present in barley grains during malting.

Proteases are vital components in the malting process as not only do they provide yeast with a source of free nitrogen, sulphur and amino acids, but they are also important contributors to the clarity, flavour and filterability of both whisky and beer, and are also involved beer foam stability and haze formation (Bamforth, 2009). Their involvement in these processes is a direct result of their roles in storage protein solubilisation (Robinson et al, 2007) as it is this process which releases the proteins and amino acids which effect these aspects of beer and whisky quality. Furthermore, protease inhibitors such as the serine protease inhibitor Protein Z, have been implicated as important components of beer foam stability (Evans et al, 1999). Thus a full knowledge of the protease spectrum of the germinating barley grain is an important and potentially valuable tool for the brewing and distilling industries as not only could it result in the production of higher quality products but also in possible changes to the malting process which could ultimately result in more cost effective malting practices.

1.6.1: Effects of Kilning on Barley Malt Endoprotease Activity

It has been claimed that the high temperatures involved in the process of kilning results in the inactivation of proteolytic enzymes (Jones, et al, 2000). However, it has been shown that this is not the case as comparative studies of the proteolytic activities of green malt and malt kilned using temperatures up to 85 ^{o}C have shown that there is no measurable effect on the levels of proteolysis between the two malts (Jones, et al, 2000) indicating that kilning does not lead to protease inactivation within the grain, and that at 85^{o}C (the temperature used by many American commercial maltsters) the kilning process can even result in an increase in the levels of protease activity within the grains (Jones, et al, 2000).

1.6.2: Barley Cysteine Proteases

Cysteine proteases are found in all tissues of higher plants, where they are involved in not only storage protein degradation and formation (through hydrolysis and post – transcriptional modifications) but also in apoptosis and organ senescence (aging), responses to oxidative stress, cell cycle control, flower development, morphogenesis and embryogenesis (Salas et al, 2008). As a consequence a large amount of research has been dedicated over the years to the roles and identities of cysteine proteases in many plant species including cereal crops.

In general, barley grain cysteine proteases have been shown to be optimally active at pH 4.8 and are located in the endosperm, embryo and aleurone tissues during germination (Zhang & Jones, 1995) and have been shown to achieve their maximum levels of activity by approximately day four of germination.

One of the earliest plant cysteine proteases to be discovered was papain. This enzyme was purified from the papaya plant, *Carica papaya* (Balls et al, 1937). Orthologs of this protease have been discovered in many other plants including rice, barley and wheat where papain – like cysteine proteases have been shown to be very important in storage protein maturation during grain development, and in the subsequent breakdown of these proteins during grain germination. For instance, in wheat, a group of papain – like cysteine proteases has

recently been discovered (Kiyosaki et al, 2009). This group consisted of triticain α, triticain β and triticain γ. All three proteases were found to be expressed during early germination in response to GA signalling, with triticain β being expressed at the onset of germination, and triticains α and γ appearing strongly from day two of germination, and also during early grain maturation (Kiyosaki et all, 2009). Triticain γ is most probably stored in the vacuole of aleurone cells due to the presence of a vacuole – sorting signal in its protein sequence. Although no literature could be found in support, it could be postulated that triticain γ is released into the endosperm via the apoptosis of aleurone cells which has been shown to occur during germination in response to GA signalling (Fath et al, 2000), but further studies would need to be carried out to support this. It was also demonstrated that triticain α, β and λ are involved in the breakdown of storage proteins during endosperm modification, and that due to their differing temporal expression patterns they may fulfil other varying roles in wheat grain germination and also maturation (Kiyosaki et al, 2009). Given the close relationship between wheat and barley, it is probable that these proteases are also present in the barley grain aleurone. A small number of papain – like cysteine proteases have also been characterised in barley seeds. Endoprotease – A (EPA) and endoprotease – B (EPB) are two such examples (Hammerton & Ho, 1986, Koehler & Ho, 1990) and came to light during studies initially carried out to investigate the effects of GA_3 on the expression of both carboxylases and proteases in isolated aleurone layers during barley grain germination (Hammerton & Ho, 1986). This study found that unlike the carboxylases investigated (which were produced independently of GA_3 treatment), GA_3 treatment was required for the production and secretion of protease activities by the aleurone layer in a time frame similar to that of α – amylase (an enzyme produced in response to GA signalling). EPA was then purified and characterised first and was shown to be 37 KDa in size, with an optimal pH of 4.5 to 5.0, an optimum temperature of 45 ^{o}C, and that it is inhibited by the cysteine protease class specific inhibitors E – 64, iodoacetate and *p* – hydroxymeruribenzoate (Hammerton & Ho, 1986). Hammerton and Ho also showed that EPA was active against the storage proteins hordein (from barley) and gliadin (from wheat). Later, three further isozymes of EPA were discovered (Koehler & Ho, 1988), all produced in response to GA signalling, and all active against the major storage proteins, hordein and gliadin providing further evidence for both the role of cysteine proteases in the mobilisation of grain storage proteins and of the control of this mobilisation by GA_3. The discovery and purification of EPA also yielded the discovery of EPB (Koehler & Ho, 1990). EPB was found to have a 90 % amino acid sequence homology and similar characteristics to that of EPA, with its size (being 30 KDa as

opposed to the 37 KDa of EPA), number of isoforms (only two compared to three for EPA) and the relative abundance (EPB is more abundant) being the only major differences between the two proteases. Later research involving mRNA localisation studies showed the presence of EPB (Jones, 2005) to be initially in the scutella epithelia and aleurone cells that stand adjacent to the embryo (after one to two days of germination) and over time spreading along the entire length of the barley grain mirroring the diffusion of GA from the embryo and along the grain (section 1.2.5). The expression of EPA & EPB at the early stages of barley grain germination, the relatively high abundance in the grain of EPB, and also their hormonal control by GA_3 indicates that endoproteases A and B may be two of the major players in storage protein hydrolysis and thus very important in determining the malting quality of barley grains.

Recently, cDNA studies have identified a further class of barley grain cysteine proteases, the cathepsin – like cysteine proteases (Martinez et al, 2003) which have been shown to be expressed in the developing endosperm and also in the aleurone layer of germinating grains in response to GA signalling. These enzymes are thought to have roles in both storage protein deposition during grain maturation and in their subsequent mobilisation during grain germination.

However, the identification and characterisation of barley grain cysteine proteases has not been without some level of confusion most probably caused by the presence of multiple isoforms of already characterised barley grain cysteine proteases. A good example of this is the identification of Malt Endoprotease – 1 (MEP- 1) (Jones, 2005) which was found after the discovery of EPA and EPB. When MEP – 1 was first discovered it was thought to be a novel enzyme, but after it was sequenced and functionally characterised it was discovered to be an isoform of EPB (Jones, 2005). A similar confusion also arose around the isolation of two further novel barley cysteine protease, the “31 KDa” protease purified by Zhang and Jones in 1996 and the “30 KDa” protease purified by Poulle and Jones in 1988 (Jones, 2005). Both of these proteases were independently isolated and thought to be novel; however when they were analysed by SDS – PAGE, the size of their protein bands and that of EPB were very similar suggesting that these two proteases were also isoforms of EPB (Jones, 2005).

Not all barley cysteine proteases are synthesised *de novo* during germination, some (and some aspartic proteases) are already present in the mature grain and are stored in specially designed organelles within the aleurone layer (protein storage vacuoles) and are released into the endosperm during germination in response to GA signalling (Bethke et al, 1996). Enzymes from these protein storage vacuoles have been implicated in the mobilisation of the grains protein stores during germination.

Although the bulk of storage protein breakdown during barley grain germination is thought to be carried out by the cysteine class endoproteases (Jones, 2005) the involvement of the members of the other protease classes is also important.

1.6.3: Barley Metalloproteases

Of the metalloproteases present in germinating barley comparatively little is known. It has been described that these enzymes are optimally active at pH seven to eight, that their activities can be detected from day one of germination and that they reach their maximum level of activity by day two (Jones, 2005). As with the cysteine proteases, the activity of barley metalloproteases have been observed in the aleurone, embryo and starchy endosperm (Zhang & Jones, 1995) and their synthesis is subject to regulation by GA. Further, it has been shown that metalloproteases play an important part in the hydrolysis of storage proteins during the malting process (Jones, 2005).

In 2005 Ogbonna and Okolo purified a 35 KDa metalloprotease from sorghum malt. Despite this metalloprotease being discovered in sorghum and not barley, it shares many characteristics with that of the barley metalloproteases (for example it has a pH optima of seven), and since sorghum and barley are both members of the cereal family it is likely that similar metalloproteases exist in malting barley grains.

1.6.4: Barley Aspartic Proteases

Knowledge surrounding the roles and identity of the aspartic class proteases in barley is limited. Aspartic class protease activities have so far been found localised to the aleurone layer and starchy endosperm only (Zhang & Jones, 1995) and are thought not to be major components of storage protein solubilisation during germination as they have been shown not to digest purified barley hordeins (Jones, 2005). However, it has been shown recently in rice (Chen et al, 2009) that there are at least 20 aspartate protease – encoding genes that are highly expressed in the germinating rice grain indicating a potential role for this enzyme class in rice germination. This role could take the form of storage protein breakdown, the activation of other enzymes such as β – amylase or in housekeeping functions, further work is required to elucidate these potential roles. Since barley and rice are very similar and that the rice genome can be used as a comparison for the barley genome, it is highly likely that there exists a similar situation within the barley grains with regards to aspartate gene expression.

1.6.5: Barley Serine Proteases

As yet only two barley grain serine proteases have so far been purified (Jones, 2005); a subtilisin – like serine protease termed serine endoprotease – 1 (SEP – 1) and Hordolisin. Both these proteases have been shown to be inactive against purified hordeins showing that the roles of these enzymes are more likely to be regulatory rather than in the solubisation of grain storage proteins. Furthermore, the elucidation of a potential role for serine class proteases in the hydrolytic breakdown of β – amylase (Schmitt & Marinac, 2008) provides further evidence for their more regulatory roles. However, a role in storage protein degradation should not be totally excluded as in wheat there exists thiocalsin, a calcium dependent thioredoxin – linked serine protease which is specific for the degradation of the wheat storage proteins, glutenins and gliadins (Besse et al, 1996), hence a similar role for yet unidentified barley serine proteases could exist.

1.7: Barley Grain Proteases – Their Endogenous Inhibitors

The study of barley grain proteases and their activities during malting and germination in crude extracts is made complex because barley grains also contain endogenous protease inhibitors which can mask the presence of protease activities within the grain (Jones, 2005). These inhibitor molecules are not only present during grain germination, but are also active in the developing and mature grain functioning to repress protein degradation until the onset of germination (Davy et al, 1999). Furthermore, within the grain there are endogenous inhibitors of microbial and animal proteases which function to protect the plant from pathogens and pests (Mikola & Enari, 1970).

The endogenous inhibitors of barley endoproteases consist of highly stable proteins which strongly bind to and switch off the activity of their target proteases (Jones, 2005). These inhibitor molecules are not just important as they can mask the activities of proteases during studies, but they are important as they contribute toward the levels of peptides, proteins and amino acids within the malt thus affecting its quality and that of the final product (Jones, 1995). Thus an understanding of the identity and biochemistry of these proteins is an important part of developing and improving malt and malting barley varieties.

It was originally postulated that the appearance and increase in activity of barley proteases at the onset of germination was due to the destruction of their endogenous inhibitors (Mikola & Enari, 1970). However, it was later shown that this was not the case as it is not the inhibitors that change in amount, but the proteases themselves (Jones, 2001). Indeed, the levels of endogenous protease inhibitors in malting and germinating grains remains unchanged from their pre – germination levels, indicating that the increase in proteolytic activity at the onset of germination is most likely to be a result of increased *de novo* synthesis and release of proteases (in response to GA signalling), changes in the redox state of grain or grain compartments which may affect the binding of protease inhibitors to their target proteases, and not a decrease in the amounts of endogenous inhibitors present within the grain.

Endogenous inhibitors of all four protease classes are thought to exist within the grain with the inhibitors of the serine and cysteine class proteases being the best characterised (Jones, 2005). As yet no metalloprotease or aspartate protease inhibitors have yet been identified within the barley grain.

1.7.1: Endogenous Inhibitors of the Cysteine Class Barley Proteases

A number of endogenous inhibitors of the cysteine class proteases have so far been identified. These include Lipid Transfer Protein 1 (LTP1), and Lipid Transfer Protein 2 (LTP2) (Jones, 1995) and members of the cystatin gene family (Martinez et al, 2009). LTP1 is a 9.7 KDa protein produced by the barley grain aleurone layer and is present in mature and germinating grains (Davy et al, 1999). This protein has been shown to have an important role in beer foam formation most likely through its competitive inhibition of EPB thus protecting important foam – promoting proteins and polypeptides from degradation. Despite this important role LTP1 is probably not active in its native conformation (Davy et al, 1999) as it is activated after undergoing extensive denaturation during wort boiling, thus it is unlikely to play a role in the modulation of endoprotease activity in either mature or germinating barley grains. This has led to a debate over the classification of LTP1 as an endogenous inhibitor since it is not active in its native state (Davey, et al, 1999). However, it should be noted that multiple isoforms of LTP1 are known to exist in barley and in its close relative wheat, leading to the suggestion that one or more of these isoforms may indeed be active in mature and / or malting barley grains (Jones, 2005).

The second LTP protein, termed LTP2 (Jones, 1995) is a 7.112 KDa in size and is synthesised in the aleurone layer of barley grains, and has been shown to inhibit the activities of cysteine class endoproteases during both two – dimensional native gel electrophoresis (with gelatin incorporated in the gels to act as the endoprotease substrate) and in *in vitro* enzyme activity studies (Jones & Marinac, 2000). This inhibitor was found (by mass spectroscopy) to be the protein encoded by an earlier cloned and sequenced barley grain gene, B11E (Jones, 2005). LTP2 mRNA has been shown to be expressed in developing but not mature barley grains, suggesting a possible function for LTP2 in the repression of protein degradation during grain filling and storage protein deposition.

Cystatins are proteinaceous inhibitors of the papain C1A family of proteinaceous inhibitors (Martinez et al, 2009). This inhibitor family was first described, in plants, in rice grains (Kondo et al, 1990) where they were termed the oryzacystatins. The oryzacystatins so far include two members, oryzacystatin – 1 (OC – 1) and oryzacystatin – 2 (OC – 2), both located in the endosperm of developing rice grains where they are thought to be involved in the regulation of grain filling and storage protein deposition. These inhibitors have also been found in barley grains (Martinez et al, 2009) where they inhibit the cathepsin L – like cysteine proteases. mRNA localisation studies have shown that these inhibitors are present in the developing endosperm and embryo, and also in the germinating embryo where they are thought to play a role in storage protein deposition during grain development, and protein mobilisation during grain germination.

1.7.2: Endogenous Inhibitors of the Serine Class Barley Proteases

Members of the trypsin / alpha amylase inhibitor family (also called the chloroform / methanol soluble protein family (CM proteins)) have been shown to be involved in the inhibition of barley grain serine proteases (Jones & Fontanini, 2003). These proteins were found to be specific inhibitors of the serine protease SEP – 1, and have putative roles in the regulation of storage protein breakdown. Recently this protein family has also been implicated in the formation and stability of haze in beer aging (Robinson et al, 2007), a role that could be brought about by the inhibition of the breakdown of small molecular weight proteins possibly by the serine class proteases.

Protein Z is a beer foam – positive protein and has been found to be an endogenous inhibitor of the serine class proteases (Hejgaard et al, 1985). Protein Z is a member of the α_1 family of trypsin inhibitors and has been postulated to function as a storage protein during grain filling, contributing a large proportion of the grains stores of the amino acid lysine. During grain germination protein Z is activated (in a similar mechanism to that of β – amylase, see section 1.8.4) by the action of cysteine class proteases yielding an active serine protease inhibitor (Guerin et al, 1992).

1.8: Starch Breakdown and Protease Activity; α– Amylase, β – Amylase, Limit Dextrinase

Starch breakdown is a process fundamental to mashing and germination as it provides yeast and the embryo respectively with a source of readily digestible carbohydrate. The processes of starch breakdown are complex, highly regulated and as yet not fully elucidated (Kotting et al, 2010). However, they are known to involve such regulatory elements as the redox state of the grain and grain compartments, protein phosphorylation, protein complex formation and proteolysis. The influence of plant growth hormones such as GA on the activity of starch degrading enzymes has been recognised for some time (Paleg, 1960 a; Paleg, 1960 b) and GA is known to be a key activator of starch degrading enzyme activity in germinating barley grains (Varner, 1964).

The diastatic potential of barley (the sum total of the activity of starch degrading enzymes within the grain) is a very important factor in determining its quality and thus suitability for malting (Arends et al, 1995). Grains with a high diastatic power are of higher quality producing malt with well modified starch and thus yielding more small carbohydrates for yeast to utilise during fermentation. A number of hydrolytic enzymes have been implicated as contributors to this measurement including α – glucosidase, α and β – amylase, and limit dextrinase (Arends et al, 1995).

1.8.1: α– Amylase

α – amylase (1, 4 – α – D – glucanohydrolase, EC 3.2.1) is one of the only known enzymes to be present in germinating barley grains which has the capacity to initiate native starch hydrolysis (Georg – Kraemer et al, 2001). The synthesis of α – amylase occurs in the aleurone layer during grain germination in response to GA signalling. Once synthesised, α – amylase is then secreted into the endosperm where it initiates starch breakdown through the hydrolysis of α – 1, 4 – linked glycosidic bonds in the starch granules liberating small molecular weight sugars such as glucose and higher dextrins which can, in turn be broken down by other starch degrading enzymes such as β – amylase, limit dextrinase and α - glucosidase. (Georg – Kraemer et al, 2001). It has been found however, that a small amount of α – amylase activity does occur during grain development indicating a possible

role for α – amylase in the regulation of starch grain formation. The presence of α – amylase at different times during the grain's life cycle implies the existence of at least two forms of α – amylase in the grain which may differ in their temporal or spatial expression patterns. Indeed, a number of different isozymes of α – amylase have been identified in barley, with their numbers depending upon the cultivar studied and how sensitive the method of detection was (Muralikrishna & Nirmala, 2005). Despite their varying numbers, all α – amylase isoforms can be identified as belonging to one of two main groups delineated by their requirements for calcium and their pI. The high pI isoforms are calcium independent, have pI values close to 5.8 and are the first α – amylases to be expressed during early germination. In contrast, the activity of the low pI isoforms are calcium ion dependent (where Ca^{2+} ions are thought to interact with negatively charged amino acid residues within the protein, such as glutamate or aspartate, thereby stabilising it and aiding in the maintenance of its conformational shape), appear later in germination and have pI values of around 4.5 (Muralikrishna & Nirmala, 2005). Both groups are synthesised by the aleurone (and possibly the scutellum) in response to GA, with the low pI isoforms accounting for up to 60 % of the total α – amylase activity during germination (Muralikrishna & Nirmala, 2005).

1.8.2: Gibberellic Acid and the Activation of α – Amylase Synthesis

As already mentioned, the synthesis of α – amylase is activated during germination by GA signalling. In section 1.2.5 it was described how at the onset of germination GA produced by the embryo diffuses out into the scutellum and the aleurone switching on the production of proteases, α – amylase and other hydrolases. An important component of the GA signalling pathway is the transcription factor HvGAMYB (Gubler et al, 1995). HvGAMYB is the transcription factor responsible for the transactivation of the high pI α – amylase genes during germination. Evidence for this role came from functional analysis studies of the promoter of the high pI α – amylase genes (Gubler et al, 1995). This study showed that the α – amylase promoter element contained a nucleotide sequence (TAACAAA) which is specifically bound to by the HvGAMYB transcription factor, and that the expression of HvGAMYB is up regulated in the presence of GA. Moreover, it was shown that the GA induced expression of HVGAMYB is part of the early responses to GA preceding that of hydrolase production. Further studies highlighted the existence of the same promoter sequence, TAACAAA, in other GA responsive genes and showed that (Murray et al, 2006)

when the expression of HvGAMYB is up regulated (by the insertion of extra copies of the HvGAMYB gene into barley lines) the production of α – amylase increases providing further evidence for its key role in α – amylase synthesis.

1.8.3: α – Amylase Inhibitors

α – amylase is synthesised in an active form during grain germination, but due to the presence of endogenous α – amylase inhibitors within the grain the levels of α – amylase activity are finely regulated. A large family of α – amylase inhibitors have been identified (Svensson et al, 2004). These are the CM (Chloroform / Methanol soluble) family of proteinaceous inhibitors and include the trypsin / alpha amylase inhibitors which are also known to inhibit the serine class proteases (section 1.5.9) (Svensson et al, 2004). These inhibitor molecules are thought to be deactivated via reduction of their cysteine residues by NADP / thioredoxin system (Kobrehel et al, 1991).

Another group of α – amylase inhibitors are members of the xylanase inhibitor family, XIP – 1 (Sancho et al, 2003). These proteins were first identified as inhibitors of xylanases but have since been shown to be active against both the high and low pI α – amylase isoforms.

1.8.4: β - Amylase

β – amylase (also called 1, 4 – α – D – glucan malto – hydrolase, EC 3.2.1.2) catalyses the release of β – maltose from the non – reducing chain ends of starch and related compounds (Dunn, 1974). Unlike α – amylase, β – amylase is not synthesised *de novo* during grain germination but accumulates in an insoluble form during grain development, complexed, through disulphide bridges, with an inhibitor protein (Georg – Kraemer et al, 2001). At the onset of germination this enzyme – inhibitor complex is broken to release free, active β – amylase. The release of β – amylase from its inhibitor has been attributed to the actions of either reducing conditions within the grain and the actions of cysteine proteases, or just the actions of cysteine class proteases alone (Grime & Briggs, 1996; Sopanen & Lauriere, 1989).

It has been shown recently that β – amylase may be degraded by serine class protease activity in a possible amino acid scavenging exercise during the later stages of germination (Schmitt & Marinac, 2008).

1.8.5: Limit Dextrinase

Limit dextrinase (amylopectin 6 – glucanohydrolase EC 3.2.1.41), sometimes also referred to as pullulanase is a starch – debranching enzyme which cleaves the (1, 6) α linkages in amylopectin and the branched dextrins produced by α and β – amylase mediated degradation of starch and its breakdown products (Ross et al, 2003). Just like α – amylase, limit dextrinase is synthesised by the aleurone layer in response to GA, and is also present at low levels in both developing and germinating barley grains. Limit dextrinase is synthesised in an inactive form bound to a proteinaceous inhibitor molecule. The activation of limit dextrinase occurs either by a combination of cysteine protease mediated breakdown of the inhibitory complex and reducing conditions or cysteine proteases alone (Longstaff & Bryce, 1993).

1.9: Objectives

This project aimed to analyse the barley grain protease spectrum during malting and germination by:

- characterising the protease activity in malted and germinating barley grains
- investigating the significance of specific proteases and protease classes in the malting process
- identifying individual proteases using the proteomic techniques of FPLC, SDS – PAGE and MALDI-TOF mass spectroscopy

2.1: Barley Malt Production

2.1.1: Vitality Studies

The barley variety used throughout this study was *Hordeum vulgare* L. cv. Oxbridge. These grains were obtained from Bairds Malt, Pentcaitland, UK. Prior to the malting (and also germination experiments, section 2.8) viability studies were undertaken to ensure that the grains were healthy following the Institute of Brewing's Recommended Methods of Analysis (Institute of Brewing and Distilling, 1991). Germinative energy studies consisted of three lots of 100 grains being selected and germinated at 18 ^{o}C on top of two 85 mm Whatman filter (Whatman, UK) papers in 90 mm diameter glass petri dishes with 4 ml of water. After three days the germination efficiency of the grains was measured by counting the number of germinating grains. An overall germination efficiency of over 97 % of grains was observed indicating that the grains were healthy and able to germinate well. A second test was also carried out, at the same time as the germinative energy studies, to ensure that the grains were not water sensitive because if they were found to be so the steeping steps would have to be broken up with periods of air drying to reduce the effects of the grain's water sensitivity on the resultant malt. Water sensitivity tests were carried out by germinating three lots of 100 grains in 8 ml of water for three days at 18 ^{o}C and counting the number of grains germinating. In all three cases the germination levels were above 75 % indicating low water sensitivity in the grains (as there was only approximately a 22 % difference in germination % between these grains and the germinative energy grains) and thus standard malting conditions could be used. The final viability test to be carried out was that of germinative capacity. This was used as a check to ensure that the grains were living even if they were not able to germinate under normal conditions due to dormancy, and was carried out by germinating three lots of 200 grains in 200 ml of 2.5 % hydrogen peroxide, then after three days at 18 ^{o}C the number of germinating grains was averaged and an overall germinative capacity of above 99 % was observed indicating that the grains were living.

2.2.2: Malt Production

Green malt was produced by micromalting (Custom Lab Micromaltings) 1 Kg of sieved (to remove grains below 2.2 mm in size and also half grains) ungerminated barley grains. Malting was carried out at 16 ^{o}C using the standard malting conditions of 8 hours steep : 16 hours rest : 24 hours steep, then germinating at 16 ^{o}C for both two and four days to produce

two and four day malt respectively. After 65 hours of malting the grains were turned to ensure even aeration and prevent root entanglement. Malt was left unkilned and frozen in 200 g aliquots until further use.

Due to enzyme stability issues encountered with the frozen green malt the decision was taken to use kilned malt due to its high stability and its ease of storage. Kilned malt was produced by kilning (in a Custom Lab Micromaltings Kiln) freshly malted four day green malt at 60 ^{o}C for 15 hours. A low kilning temperature was selected to ensure a stoppage of germination, but no loss of protease activity (Jones et al, 2000).

2.2: Green Malt Crude Extract Studies

2.2.1: Malt Crude Extract Preparation

The initial malt studies were performed using unfractionated two and four day green malt crude extracts produced following a method modified from Jones et al, (1998). Green malt was extracted in, 3 ml/g fresh weight, cold 50 mM sodium acetate buffer pH 5 containing 0.1 % Triton X-100 (Sigma, Dorset, UK) by homogenisation in a blender for approximately ten minutes. The macerated barley grains were then centrifuged twice at 4 ^{o}C, at 20000 xg for 15 minutes each time. Following centrifugation the crude extracts then underwent two rounds of vacuum filtration using firstly, 0.45 µm filters (Whatman) followed by 0.2 µm filters. Filtrated extracts were kept on ice until assayed.

2.2.2: The Bradford Protein Assay

The protein content of crude extracts and FPLC fractions was assayed using the Bradford method (Bradford, 1976) which is based upon the addition of protein to a Coomassie dye. In the presence of protein the Bradford solution will change in colour from brown to blue. The intensity of this colour change is dependent upon the protein content of the sample – the higher the protein concentration the more intense the colour change. This colour change is measured spectrophotometrically at a UV absorbance wave length of 595 nm and from this

the protein content of the sample can be determined using a calibration curve produced form known concentrations of BSA (Sigma, Dorset, UK) as standard.

In the present study 10 µl of crude extract was added to 1 ml of Bradford dye (produced in house), mixed thoroughly by inversion, and the intensity of the colour change read using a Shimadzu UV 1650 duel beam spectrophotometer (Shimadzu, UK). The resultant UV absorbance readings were used to calculate the protein content in µg/µl of the crude extract using the calibration.

2.2.3: The Azocasein Protease Assay

The protease activity of crude extracts was assayed using the protease substrate azocasein (Sigma, Dorset, UK) and a modified method from Jones et al (1998). Azocasein consists of the protein casein derivatised with an azo dye. Proteolytic cleavage of azocasein releases free azo dye into the surrounding solution, thus changing its colour. The amount of dye released is directly proportional to the amount of protease activity present in the sample and can be measured spectrophotometrically at a UV absorbance wavelength of 440 nm.

Crude extracts were assayed using the pH values and buffers listed in Table 2.1. These assay buffers contained 5 mM DTT (Sigma, Dorset, UK) unless otherwise stated. All assays were carried out in 2 ml polypropylene tubes (Greiner) at 36 ^{0}C in a constant temperature water bath unless otherwise stated.

Table 2.1: assay buffer systems

pH	Buffer
4	McIlvaine's Buffer (0.2 M sodium dihydrogen phosphate and 0.1 M citric acid)
5	100 mM sodium acetate – acetic acid
6	100 mM sodium acetate – acetic acid
7	100 mM Tris-HCl
8	100 mM Tris-HCl
9	100 mM Tris-HCl
10	100 mM Tris-HCl

Assays were carried out by adding 100 µl of crude extract to 395 µl of assay buffer along with 500 µl of 1 % w/v aqueous azocasein and 5 µl 1 mM DTT stock solution. The assays were then shaken well to ensure thorough mixing and incubated in a constant temperature water bath at 36 ^{o}C for one hour. Controls consisted of 495 µl of assay buffer, 5 µl 100 mM DTT stock solution, and 500 µl of 1 % (w/v) aqueous azocasein. Assays were stopped by the addition of 700 µl of cold 15 % (w/v) trichloroacetic acid (TCA), shaken and left on ice for at least 15 minutes to ensure full precipitation of any undigested azocasein. The assays were then centrifuged in a bench top microfuge at 13000 xg for five minutes to yield an orange supernatant containing the cleaved azo dye. After centrifugation 1 ml of supernatant from each assay was transferred to 1 ml cuvettes and analysed spectrophotometrically at the UV absorbance wavelength of 440 nm.

To increase the sensitivity of the assay a slight modification was made to the assays from the FPLC fractionations. For these, 200 µl of sample was added to 395 µL 1 % (w /v) aqueous azocasein along with 500 ul of 100 mM sodium acetate pH 5. No DTT was used in these assays as the protease classes under investigation (serine and metallo) have no known requirement for reducing conditions.

2.2.4: Class Specific Inhibitor Studies

To investigate the roles of the four different protease classes in the overall levels of protease activity of crude extracts, class specific inhibitors were used. Specifically E – 64 (N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine) (cysteine proteases), PMSF (phenylmethylsulfonyl fluoride) (serine and to a lesser extent cysteine proteases), 1, 10 – phenanthroline (metalloproteases) and pepstatin A to inhibit aspartic proteases. All inhibitors were purchased from Sigma, Dorset, UK. Class specific inhibitor studies were carried out in much the same manner as the protease activity assays previously described in section 2.2.2, with the only difference being that an appropriate quantity of class specific protease inhibitor was added to each assay buffer (Table 2.2).

Table 2.2: class specific protease inhibitors

Inhibitor	Protease Class	Solvent	Working Concentration
E – 64	Cysteine	Distilled Water	10 µM
PMSF	Serine	Ethanol	5 mM
1, 10 Phenanthroline	Metallo	Methanol	5 mM
Pepstatin A	Aspartic	Methanol	20 µM

2.3: Fast Protein Liquid Chromatography

2.3.1: Ion Exchange Chromatography

Both four day Oxbridge malt crude extracts and “cracked” grain extracts (section 2.5.1) were fractionated by ion exchange chromatography using an AKT1 - ÄKTA FPLC (GE Healthcare, Uppsala Sweden) equipped with a P – 900 pump system and a UV – 900 constant wavelength UV detector set at a UV absorbance of 280 nm. Anion exchange (HiPrep 16 / 10 Q FF) and cation exchange (HiPrep 16 / 10 SP FF) columns were both used during this study and were also supplied by GE Healthcare, Uppsala, Sweden. Both columns had a 20 ml column volume, column matrices consisting of 6 % highly cross – linked spherical agarose and a mean particle size of 90 µm. The columns were both run at a flow rate of 5 ml / minute and at a maximum system pressure of 0.25 MPa (maximum column pressure

plus system pressure) and fractions were collected using the Frac – 920 automated fraction collector (GE Healthcare, Uppsala, Sweden).

2.3.2: Size Exclusion Chromatography

During the metalloprotease enrichment (section 2.4), the ion exchange fractions exhibiting the highest levels of metalloprotease activity were concentrated using an Amicon® Ultra – 15 centrifugal filter unit (Millipore, County Cork, Ireland), with a 3 KDa lower retention limit, to reduce their combined volume to approximately 1 ml. The concentrated sample was then injected onto a size exclusion column using a 2 ml sample loop (GE HealthCare, Uppsala, Sweden). The size exclusion column used was a Superose 12 10 / 300 GL gel filtration column (GE Healthcare, Uppsala, Sweden) with a column volume of 24 ml, an average particle size of 11 µm, a globular protein molecular weight exclusion limit of approximately 2000 kDa and an optimal separation range of 1000 – 3 x 10^5 Da. The column was run at a pressure limit of 3.00 MPa and a flow rate of 0.8 ml / min. Prior to the application of any ion exchange fractions, the column was calibrated to produce a calibration curve (Fig. 2.1) from which the molecular weights of the molecules in each fraction could be approximated.

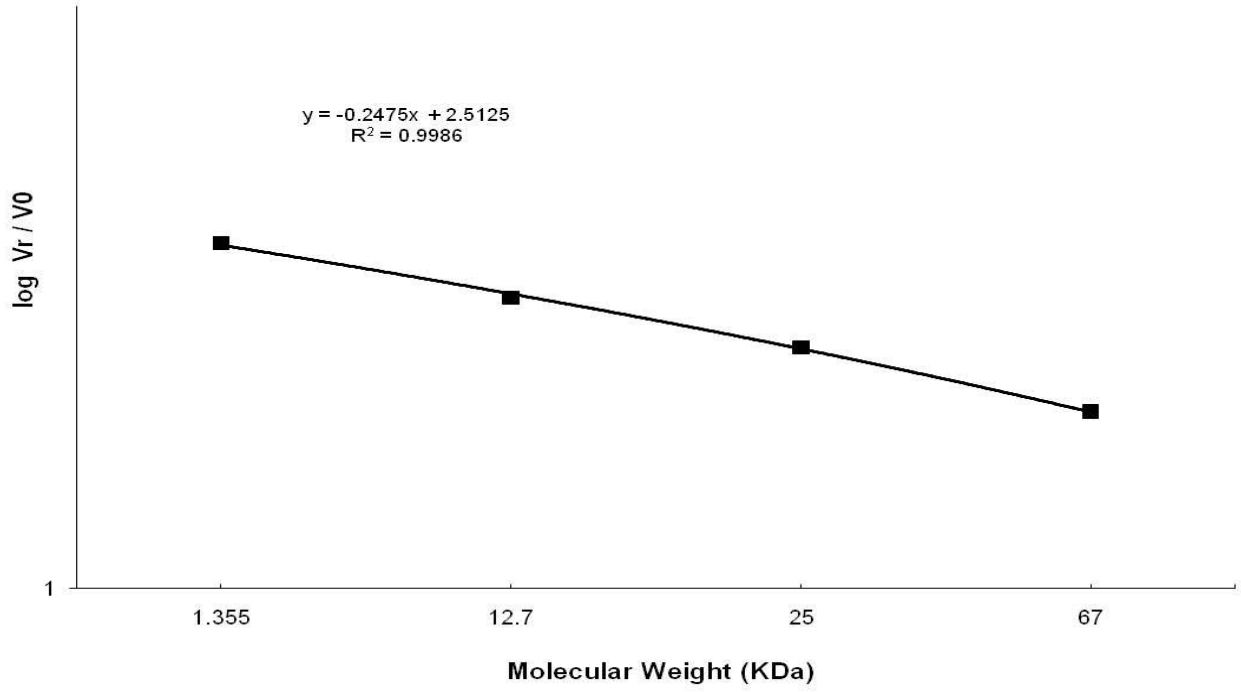


Figure 2.1: Gel Filtration Calibration

The data above shows the calibration curve for the Superose 12 10 / 300 GL gel filtration column, where log V_r / V_0 is the logarithm of the retention volume divided by the void volume (i.e. the volume it took for the protein to be eluted from the column divided by the volume it took for the unretained Blue Dextran to pass through the column).

Calibration was achieved using 10 mg/ml blue dextran (MW 2000 kDa), 8 mg/ml BSA (MW 67 kDa), 5 mg/ml chymotrypsinogen A (Mw 25 kDa), 1 mg / ml cytochrome C (MW 12.7 kDa) and 0.3 mg/ml vitamin B_{12} (MW 1.355 kDa). Blue dextran, having a large molecular weight, thus passed directly through the column, and was used for void volume determination. The column was equilibrated using 1.5 column volumes of buffer A. Due to the nature of size exclusion separation (i.e. separation being achieved by way of molecular size not charge) no salt gradient was required for sample elution, thus the elution step consisted of 1.5 column volumes of buffer A. The fraction size used was 1ml.

2.4: Metalloprotease Enrichment

The enrichment of metalloproteases was carried out following the process outlined, below in Fig. 2.2.

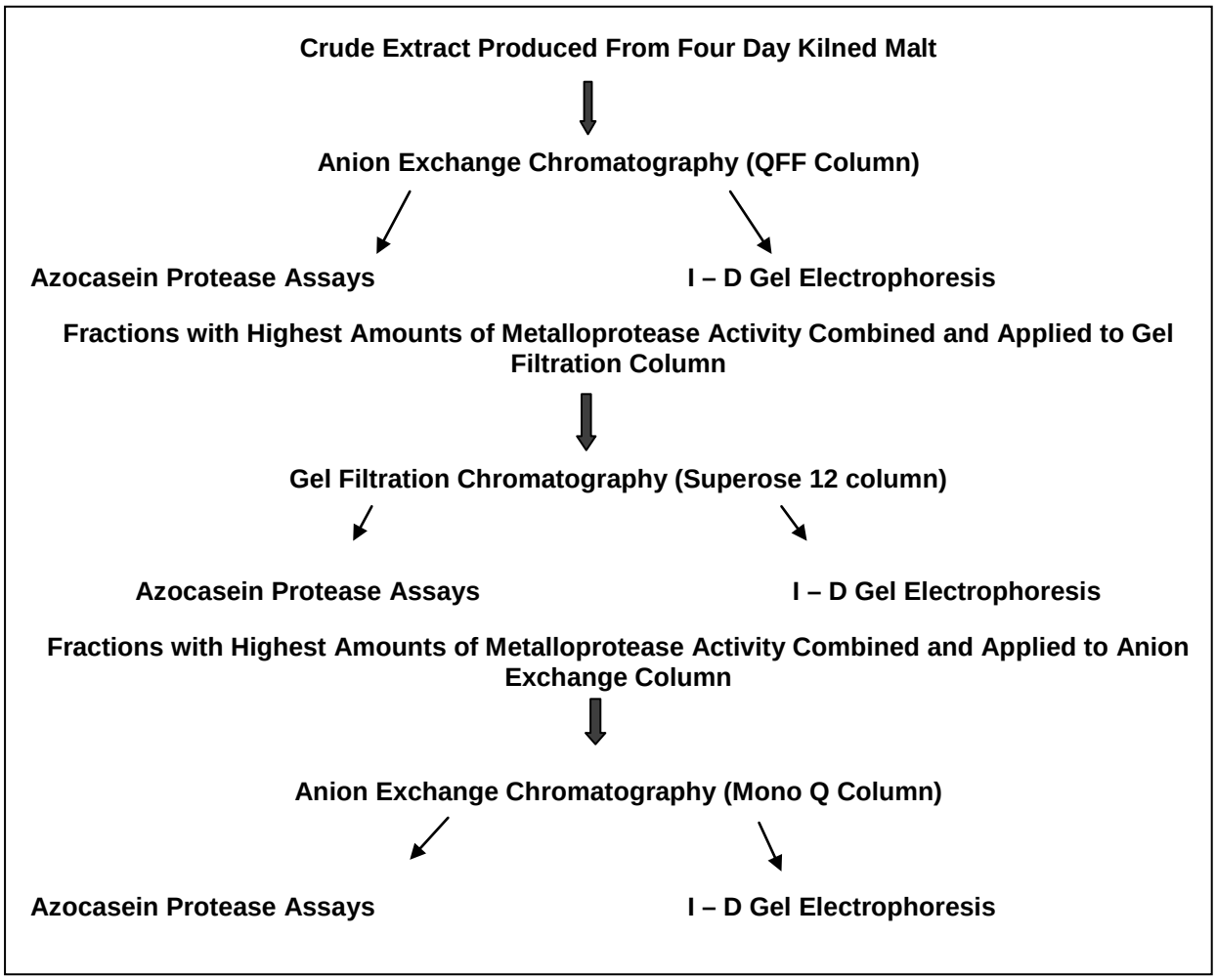


Figure 2.2: Metalloprotease Enrichment Procedure

2.4.1: Anion Exchange and Gel Filtration Chromatography

The HiPrep 16 / 10 Q FF anion exchange column (GE Healthcare, Uppsala, Sweden) was used for the first step in the purification of metalloproteases from four day kilned malt (extracted following the method described in section 2.2.1) The column was equilibrated using five column volumes of buffer A (50 mM Tris-HCl plus 50 mM NaCl pH 8.0) followed by five column volumes of buffer B (50 mM Tris-HCl plus 2 M NaCl pH 8.0) then five more column volumes of buffer A. Following equilibration approximately 70 ml to 80 ml of crude extract was pumped onto the column via the buffer A pump. Elution was achieved using a segmented gradient of 0 – 60 % buffer B over three column volumes, collecting 3 ml fractions, then 60 – 100% buffer B over three column volumes, again collecting 3 ml fractions. The fraction numbers corresponding to the chromatographic peaks were assayed

for both protein content (Bradford assay, section 2.2.2) and metalloprotease activity using the following assay method: 150 µL 1 % (w/v) aqueous azocasein, 150 µL fraction and 150 µL assay buffer (buffer A) as a positive control, and the same volumes of each for the inhibitor containing assays except that 5 µL of buffer was substituted for 5 µL of 500 mM 1, 10 phenanthroline stock solution to produce a final concentration of 5 mM 1, 10 phenanthroline in the assay. The control was 295 µl buffer A, 5 µL of 500 mM 1, 10 phenanthroline stock solution and 150 µL 1 % (w/v) aqueous azocasein. Those fractions exhibiting the highest levels of metalloprotease activity were combined (after taking a sample for running on SDS – PAGE gel electrophoresis – section 2.6.1) and reduced in volume (using Amicon® Ultra – 15 centrifugal filter units as described above) and loaded onto the equilibrated Superose 12 10 / 300 GL gel filtration column using the method outlined in section 2.3 2. This gel filtration step was used as not only a further separation step but also as way of desalting the pooled fractions before loading them onto the Mono Q anion exchange column. If the pooled fractions had maintained their post HiPrep 16 / 10 QFF column salt levels no proteins would have bound to the Mono Q column.

Following gel filtration, those fractions corresponding to the chromatographic peaks were again assayed for both protein content and metalloprotease activity and those with the highest levels of metalloprotease activity were again pooled (following sampling for SDS – PAGE gel electrophoresis – section 2.6.1) and this time loaded onto a Mono Q 5 / 50 GL anion exchange column (GE HealthCare, Uppsala, Sweden).

2.4.2: Mono Q Anion Exchange

The Mono Q 5 / 50 GL anion exchange column was used as a third step in the purification process as it is of higher resolution than both the QFF and Superose 12 columns. The Mono Q column had a polystyrene matrix, a mean particle size of 10 µm and an average loading capacity of 50 mg of protein. The column volume was 1 ml and it was ran at a flow rate of 2 ml / minute and at a maximum system pressure of 4 MPa. The Mono Q column was equilibrated using five column volumes of buffer A, followed by five column volumes of buffer B, then five further column volumes of buffer A. Buffers A and B were the same as used for the HiPrep 16 / 10 QFF column (section 2.4.1). Again the fractions corresponding to the protein peak were assayed for both protein content and metalloprotease activity, and

those fractions exhibiting metalloprotease activity were further separated using SDS – PAGE gel electrophoresis (section 2.6.1). SDS – PAGE gel electrophoresis analysis was carried out after each step of purification as a check on the efficiency of the FPLC separation process.

2.4.3: Ammonium Sulphate Precipitation

In an attempt to reduce the number of interfering proteins present during the metalloprotease enrichment process ammonium sulphate precipitation was used. To 10 ml samples of four day malt crude extract increasing concentrations (20 to 100 % saturation) of ammonium sulphate (Sigma, UK) were added by dissolving (at 4 ^{o}C and continuous stirring) small quantities of ammonium sulphate until the desired concentration was reached. The ammonium sulphate was added in a step – wise manner to prevent the possible formation of pockets of undesirably high salt concentrations. Once the ammonium sulphate was fully dissolved, the solution was left for 60 minutes at 4 ^{o}C to ensure enough time for full protein precipitation to occur. The samples were then centrifuged at 13 000 xg for 15 minutes to produce a pellet of precipitated protein. The supernatant was then carefully removed using a Pasteur pipette (the supernatants were also assayed for metalloprotease activity) and the pellet dissolved in 10 mL buffer A and assayed (along with samples of the original four day malt crude extract) for both metalloprotease activity and protein content.

2.4.4: HiPrep 26 / 10 Desalting Column

The HiPrep 26 / 10 desalting column (GE Healthcare, Uppsala, Sweden) was used as a method of removing the ammonium sulphate salt (which would prevent protein binding on the ion exchange columns) from the ammonium sulphate fractionated four day malt crude extracts. The desalting column has a Sephadex™ matrix composed of finely cross – linked dextran, with an average particle size of 90 µm and a protein exclusion limit of 5000 kDa. The column volume was 53 ml, with a 15 ml void volume. The column was ran at 10 ml / min at a maximum system pressure of 2 MPa, and the buffer used was the same as buffer A used for the HiPrep 16 / 10 QFF column (section 2.4.1). FPLC fractions corresponding to the protein peak were assayed for both protein content and metalloprotease activity.

2.5: Serine Protease Enrichment

2.5.1: Extract Preparation

For the enrichment of serine class proteases a different extract preparation procedure was implemented. This procedure was adapted from Koehler & Ho (1988) and involved the germination of "cracked" ungerminated Oxbridge barley grains in two 500 ml conical flasks containing 100 ml of extraction buffer (20 mM sodium succinate + 20 mM calcium carbonate + 25 μM GA_3 pH 5.0). Each flask held 1000 grains, was incubated at a constant temperature of 20^{o}C with constant agitation at 200 rpm in a constant temperature orbital incubator shaker (Gallenkamp, Germany). The extraction buffer was changed every 24 hours. The grains were initially left whole to initiate germination however, following the first 24 hour incubation period the grains were "cracked" by cutting them in half using a scalpel – before being returned to the incubator. The extraction buffer was changed every 24 hours with the dual aim of collecting compounds that the grains released during that day of germination and also minimising potentially interfering proteins and other biomolecules.

2.5.2: Serine Proteases and β – Amylase

To investigate the role of serine proteases in the degradation of β – amylase and to decide which day of extraction to take samples from for separation on the FPLC, extracts from days one to four of the process described above were investigated for their protease activity in the presence of different class specific protease inhibitor combinations (section 2.2.3 for inhibitor concentrations). The influence of the extracts on the activity of commercially acquired pure β – amylase (Sigma, Dorset UK) was also investigated. This was achieved by carrying out the β – amylase activity assay (section 2.7) with pure β – amylase at a dilution of 1 mg/ml (= 1 unit of activity/ml), and adding different volumes of extract from the day of extraction which exhibited the highest levels of serine protease activity (and the same volumes of extraction buffer as a control) to the assay.

2.5.3: Cation Exchange Chromatography

The HiPrep 16 / 10 SP FF cation exchange column (GE Healthcare, Uppsala, Sweden) was used for (following the method described in section 2.3.1) the purification of serine class

proteases from the "cracked" extract day (2.5.1) which exhibited the highest levels of serine protease activity. The column was equilibrated using five column volumes of buffer A (50 mM sodium succinate pH 5.0) followed by five column volumes of buffer B (50 mM sodium succinate + 2 M NaCl pH 5.0) then five more column volumes of buffer A. Following equilibration approximately 170 ml to 180 ml of crude extract was pumped onto the column via the buffer A pump. Elution was achieved using a continuous gradient of 0 – 100 % buffer B over five column volumes, collecting 5 ml fractions. The fraction numbers corresponding to the chromatographic peaks were assayed for both protein content (Bradford assay, section 2.2.2) and serine class protease activity using the following assay method: 300 µL 1 % (w/v) aqueous azocasein, 100 µL fraction and 300 µL assay buffer (buffer A) as a control and the same volumes of each for the inhibitor containing assays except that 35 µL of buffer was substituted for 35 µL of 100 mM PMSF stock solution to produce a final concentration of 5 mM PMSF in the assay. Samples of the fractions containing serine protease activity were fractioned on an SDS – PAGE gel following the method described in section 2.6.1.

2.5.4: Matrix – Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MaldiToF)

2.5.5: In – Gel Digestion of Protein Bands

Bands were selected from the SDS – PAGE gel to be analysed by mass spectroscopy in an attempt to identify proteins from the FPLC fractions. Samples were prepared for analysis following the Trypsin Gold, Mass Spectrometry Grade Instruction Protocol (Promega, USA). The SDS – PAGE gel bands to be analysed by mass spectroscopy were cut out of the gel using a spot picker, with a 1.5 mm tip (The Gel Company, USA), and placed into pre washed (washed with 50 % acetonitrile (ACN) in 0.1 % trifluoroacetic acid (TFA)) 0.5 ml microcentrifuge tubes and destained (to remove the Coomassie stain) two times in 0.2 ml 100 mM ammonium bicarbonate in 50 % ACN. Each destain process was carried out at 37 ^{o}C for 45 minutes. Following destaining the gels slices were then dehydrated for five minutes at room temperature in 100 µL of 100 % ACN. Gels slices were fully dehydrated when they appeared whitish / opaque in colour. The ACN was then removed from the dried slices by further drying them in a SpeedVac® (ThermoSavant) for one hour at room temperature.

2.5.6: Trypsin In – Gel Digestion

Once fully dried and all the ACN was evaporated off, the gel slices then underwent digestion by incubating them for one hour at room temperature in a 20 µg/ml solution of Trypsin Gold (Promega, USA) in digestion buffer (40 mM ammonium bicarbonate + 10 % ACN). Next more digestion buffer was added to the gel slices so that they were completely covered in buffer and the slices were then incubated at 37 ^{o}C overnight.

2.5.7: Peptide Extraction - ZipTips®

The following day 150 µL of Milli - Q® (Millipore, UK) water was added to the gels slices and they were incubated at room temperature for 10 minutes with continuous vortexing. The liquid was then carefully decanted from the gel slices and saved in fresh microcentrifuge tubes (a separate fresh tube for each gel slice). Next the gel slices were extracted two times in 50 µL of 50 % ACN / 5 % TFA extraction buffer. Both extraction procedures were carried out at room temperature, with continuous vortexing and for 60 minutes each time. After each extraction procedure the liquid was carefully decanted and pooled with that from the digestion stage. The pooled extracts were then dried, at room temperature, in a SpeedVac® (Thermo Scientific, UK) for three to four hours (or until completely dried) to eliminate the liquid and thus concentrate the peptides within. The peptides were then purified using ZipTip® pipette tips (Millipore, UK) following the manufacturer's instructions. ZipTips® are 10 µL pipette tips containing a fixed chromatography media (C_{18}) that can be used to purify and concentrate small peptides from solution. Before the pooled and dried gel slice solutions could be passed though the ZipTip®, the ZipTip® chromatographic bed had to be prepared. This was carried out by washing it (i.e. pipetting though it) once with 10 µL of 100 % ACN, then three times with 10 µL of 0.1 % (v/v) TFA in Milli - Q® water. This preparation step was also used to wash the ZipTips® between dried pooled samples. Each ZipTip® could be washed and reused up to three times. The dried pooled extracts were reconstituted for purification using the ZipTip® through the addition of 20 µL 0.1 % TFA in NANOpure® water. The reconstituted extracts were loaded onto the ZipTips® by pipetting them fully into and out of the tips five to six times, fully expelling the liquid each time. Once loaded the ZipTips® were washed two to three times by pipetting (fresh each time) 10 µL of 0.1 % TFA in NANOpure® water though each tip. This helped to remove any unbound peptides and other potential contaminants from the bound samples. The ZipTips® were then eluted and spotted

directly on the MALDI plates using 2.5 µL of 70 % ACN + 0.1 % TFA + 10 mg/ml α – cyano – 4 hydroxycinnamic acid (the matrix required for ion generation in the MALDI – ToF mass spectrometer).

2.5.8: Matrix – Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry

The extracted peptides then underwent Matrix – Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis using an Ettan MALDI – TOF – Pro mass spectrometer (GE Healthcare, Uppsala, Sweden) set to 20 Kv positive ion reflector mode, with the ion mass rejection set at 500, and pulsed extraction on (using a focus mass of 2500). Three spectra (250 shots) were acquired using an eight shot / second laser mode. Trypsin was used as the internal standard with its products having mass / charge ratios of 843.51 *m/z* and 2211.10 *m/z*. Protein identification was carried out using the MASCOT Peptide Mass Fingerprint database (Matrix Science Incorporated, USA).

2.6: Protein Gel Methods

2.6.1: Denaturing 1 – Dimensional Polyacrylamide Gel Electrophoresis (SDS – PAGE)

The discontinuous buffer system described by Laemmli (1970) was used. Before the gels were poured the electrophoresis apparatus was set up according to manufacturers instructions (GE Healthcare, Uppsala, Sweden). The resolving gel solution was poured in between a glass plate and an alumina backing plate, a thin layer of water saturated isobutanol was poured on top to create a flat surface, and the gel was then left to polymerise at room temperature for approximately 45 minutes. The resolving gel solution contained 375 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 10 % acrylamide (29 : 1 acrylamide : bis acrylamide used throughout), 0.1 % ammonium persulphate, and 0.005 % TEMED. Once the resolving gel was set the water – saturated isobutanol was poured off and the stacking gel was poured on top. The stacking gel consisted of 4 % acrylamide, 0.05 % ammonium persulphate, 125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS and 0.1 % TEMED. Wells were created in the stacking gel to provide a loading point for the protein samples by inserting a comb into the top of the stacking gel before it was left to polymerise or approximately 45 minutes. The gel, still in between the glass plate and back plate, was then placed into the electrophoresis

apparatus and the comb removed. The wells were then filled to the top of the plates with 1 x SDS – PAGE running buffer (25 mM Tris-HCl, 1.92 mM glycine, 0.5 % SDS). Prior to protein loading the samples were precipitated overnight at – 20 ^{o}C following the method detailed in section 2.6.2. Following precipitation the protein samples were mixed, by vortexing, with a 1 : 1 ratio of distilled water to two times gel loading buffer (60 % (v/v) glycerol, 0.15 M Tris-HCl pH 6.8, 0.09 % (w/v) bromophenol blue, 1.2 % (w/v) SDS). The samples were then heated at 95 ^{o}C for 10 minutes along with the molecular weight ladder (Sigma Molecular Weight Markers SDS6H2, M. W. 30 KDa to 200 KDa, Sigma, Dorset, UK). Following this the samples (and the molecular weight marker) were centrifuged at 13000g for 3 minutes and loaded into the wells of the stacking gel. Electrophoresis was then carried out at 150 V for approximately 45 minutes, or until the dye front had reached the bottom of the gel. Gels were then removed from between the glass plates and placed into a solution of Coomassie Brilliant Blue stain for fifteen minutes to one hour at room temperature. The gel was left on a shaker throughout to ensure even staining. The Coomassie stain consisted of 0.1 % (w/v) Coomassie brilliant blue R250, 10 % (v/v) glacial acetic acid and 50 % (v/v) methanol. Following staining the gels were then destained overnight in a solution of 10 % (v/v) glacial acetic acid and 50 % (v/v) methanol to remove the background.

2.6.2: 100 % TCA Precipitation

Fractions from FPLC fractionation, crude extracts and other barley samples to be analysed by SDS – page gel electrophoresis were precipitated using 100 % (w/v) TCA (trichloroacetic acid, Sigma, Dorset, UK) in water. Precipitation was achieved by adding equal volumes of 100 % TCA solution to a specific volume of sample (the sample volume used was enough to contain approximately 25 µg of protein) and left overnight at – 20 ^{o}C. Sample protein content was determined using the Bradford method described in section 2.2.2 prior to protein precipitation. Following precipitation the samples were centrifuged for 15 minutes at 13 000 xg in a microfuge to pellet the precipitated protein. The supernatant was then discarded and replaced with ice cold 100 % acetone and centrifuged at 13, 000 xg for 15 minutes to wash off any non precipitated protein and residual TCA. This washing procedure was carried out a total of three times. The pellets were then air dried at room temperature until the samples no longer smelled of acetone indicating that all the acetone had evaporated from the pellets. The samples were then prepared for SDS - PAGE electrophoresis (section 2.6.1).

2.6.3: Western Blotting

A protein extraction procedure was followed where 4 g of barley sample was extracted, on ice, in 4 ml of 1 x TBS – T buffer (150 mM NaCl, 65 mM Tris, 0.05 % Tween – 20). The grains were extracted for one hour, on ice, before being centrifuged for 15 minutes at 20 000 xg and their protein content measured using the Bradford assay (section 2.2.2). The samples were then separated by SDS – PAGE electrophoresis (section 2.6.1) and transferred onto a nitrocellulose membrane, with a pore size of 0.2 µM (Biorad, UK) using an EC140 Mini – Electroblot system (Thermo, UK) using the method described below. For each western blot carried out two SDS – PAGE gels were run, one to be transferred and the other to be stained with Coomassie (2.6.1) as a check to ensure that the gels had ran correctly. A different set of molecular weight markers were used for western blotting than for the SDS – PAGE gels. Instead of the unstained markers, pre – stained markers were used (PageRuler™ Plus Pre Stained Protein Ladder, Fermentas) as these can act as a further check (on top of the Ponceau staining – see later) to ensure that the transfer of proteins has occurred effectively.

The gel to be transferred was, following electrophoresis, carefully removed from in between the glass and alumina plates and equilibrated for five minutes in 1 x Towbin buffer (25 mM Tris, 192 mM glycine and 5 % (v/v) methanol) at room temperature. At the same time a section of nitrocellulose membrane was cut to a similar size as the gel and also equilibrated in the 1 x Towbin buffer. Care was taken to ensure that no contaminating protein was transferred onto the membrane by wearing gloves at all times. The Mini Blot Module cassette was set up as follows (all components were completely saturated in 1 x Towbin buffer): two pre – soaked Scotch – Brite pads (cut to fit into the cassette to prevent leakage) were placed on top of the cathode followed by two pieces of Whatman No. 1 filter paper. The SDS – PAGE gel was then placed on top of these and flooded with 1 x Towbin buffer to prevent drying. The nitrocellulose membrane was then placed onto the gel with care not to trap any air bubbles between the gel and the membrane as this would prevent protein transfer. Next, two further pieces of Whatman No.1 filter paper were placed on top of the membrane, followed by two more Scotch – Brite pads, again cut to fit snugly into the cassette. The cassette was then closed by placing the anode on top of the Scotch – Brite pads to complete the sandwich. The cassette was then placed into the blotting chamber so that the cathode side was facing the cathode electrode, and filled to the top with 1 x Towbin

buffer. Protein transfer was then carried out at constant voltage of 15 V for two hours at room temperature.

Following transfer the membrane was removed from the cassette and stained with Ponceau red stain (2 % (w/v) Ponceau S (Sigma, Dorset, UK), 50 mM glacial acetic acid) for approximately five minutes at room temperature. This staining was carried out to check the efficiency of the transfer process. The Ponceau red stain was then gently rinsed from the membrane with distilled water to reveal the stained bands. Further washes were carried out to completely remove the Ponceau red stain.

2.6.4: Detection with Rabbit Anti – α – Amylase Antibody

Following transfer the membrane was blocked for one hour at room temperature in 1 x TBS –T (150 mM NaCl, 65 mM Tris, 0.05 % (v/v) Tween – 20) containing 1 % (w/v) non – fat skimmed milk powder (Tesco, UK). The membrane was then incubated in the primary antibody at a dilution of 1 : 5000 dilution of rabbit anti – alpha – amylase antibody (Agrisera, Vännas, Sweden) in 1 x TBS – T containing 1 % non – fat skimmed milk powder over night at 4 ^{o}C. Next, the antibody solution was decanted and the membrane washed five times in 1 x TBS – T, once quickly and four times for 20 minutes each time. All washes were carried out at room temperature with gentle agitation. Following this the membrane was incubated in the secondary antibody at a dilution of 1 : 10000 goat – anti rabbit – HRP linked antibody Sigma, Dorset, UK) for one hour at room temperature. The antibody solution was then decanted and the membrane washed three times for 10 minutes each time in 500 ml of 1 x TBS – T.

2.6.5: Chemiluminescence Detection

Antibody detection was carried out using the ECL Plus Western Blotting kit (Amersham, UK) according to the manufacturer's instructions. Immediately after the final washing steps the membrane was allowed to drip dry to remove excess wash buffer and was placed (protein side up) on top of a clean piece of acetate film. Next the reagent solution (made up according to manufacturer's instructions) was pipetted on top of the membrane and the

membrane incubated for five minutes at room temperature. The excess reagent solution was then allowed to drip off the membrane (to prevent high background from the reagent itself) by holding it in forceps and touching a corner against clean tissue paper. The membrane was then placed onto a fresh piece of acetate followed by a second acetate piece on top of the membrane and all three enclosed inside an X – ray cassette. The membrane was then exposed to X – ray film. After 25 minutes the X – ray film was developed using Kodak fixer and developer solutions diluted as directed by the manufacturer. The X – ray film was placed in the developer solution and gently agitated until a signal could be seen. The film was then placed in water and briefly agitated, to remove excess developer, before being placed in the fixer solution and agitated gently for two to three minutes. The film was again rinsed in water to remove any excess fixer and left to air dry.

2.7: Amylase and Limit Dextrinase Assays

Assays of α – amylase, β – amylase and limit dextrinase were carried out on germinating and four day micro - malted and kilned Oxbridge barley grains (with and without class – specific protease inhibitors) according to manufacturers instructions using α – amylase, β – amylase and limit dextrinase assays kits (Megazyme International Ltd, Ireland). The α – amylase and β – amylase assay kits were based upon the methods of McCleary & Sheehan (1987) and Sheehan and McCleary (1988) respectively. The units of activity in each assay were defined as follows;

- α – amylase: one unit of activity = the amount of enzyme required to release one micromole of p – nitrophenol from blocked *p* – nitrophenyl maltoheptaoside in one minute, in the presence of excess thermostable α – glucosidase, under the assay conditions defined within the assay kit.

- β – amylase: one unit of activity = the amount of enzyme required to release one micromole of *p* – nitrophenol from PNPβ – G3 in one minute, in the presence of excess thermostable β – glucosidase, under the defined assay conditions.

- The activity of limit – dextrinase was determined with reference to limit – dextrizyme standard curve (Appendix) which converts absorbance values to milliunits per assay, then calculated using the following equation: Units of limit – Dextrinase activity per kg

malt = milliunits per assay * 0.001 * 32000, where 0.001 is the conversion factor from milliunits to units, and 32000 is the conversion factor from the activity per assay to the activity per kg malt.

The measurements of "free" β – amylase activity (section 3.4.4) were carried out by omitting the reducing agent cysteine from the extraction buffer during the extraction stage of the assay kit.

2.8: Germination Studies

Germination studies were carried out following the Institute of Brewing's Recommended Methods of Analysis (Institute of Brewing and Distilling, 1991) 4 ml Germinative Energy test protocol. To 100 grains, in glass petri dishes on top of two 85 mm Whatman filter papers (Whatman, UK), 4 ml of distilled water containing a different class specific protease inhibitor per petri dish (1 mM PMSF, 20 μM pepstatin – A, 5 mM 1, 10 phenanthroline and 10 μM E - 64) was added. To the control 4 ml of distilled water without any inhibitors was added. Petri dishes were set up to enable enzyme activity assays to be carried out at 24, 48, 72, 96 and 120 hours – i.e. one petri dish of grains per day per inhibitor. Germination was carried out at a constant temperature of 18 ^{o}C in a constant temperature incubator. At 72 hours of germination an additional 3 ml of distilled water (plus inhibitor) was added to each dish as a preventative measure against dehydration induced stress. Protease activity assays were carried out using 20 mM sodium succinate + 20 mM calcium carbonate pH 5.0 as both the extraction and assay buffer.

3.1: Azocasein Protease Assay Calibration

Trypsin protease activity was measured (using the azocasein protease assay), in triplicate, using a serial dilution of trypsin in order to produce a suitable calibration for approximating the units of protease activity in subsequent barley extract azocasein protease assays.

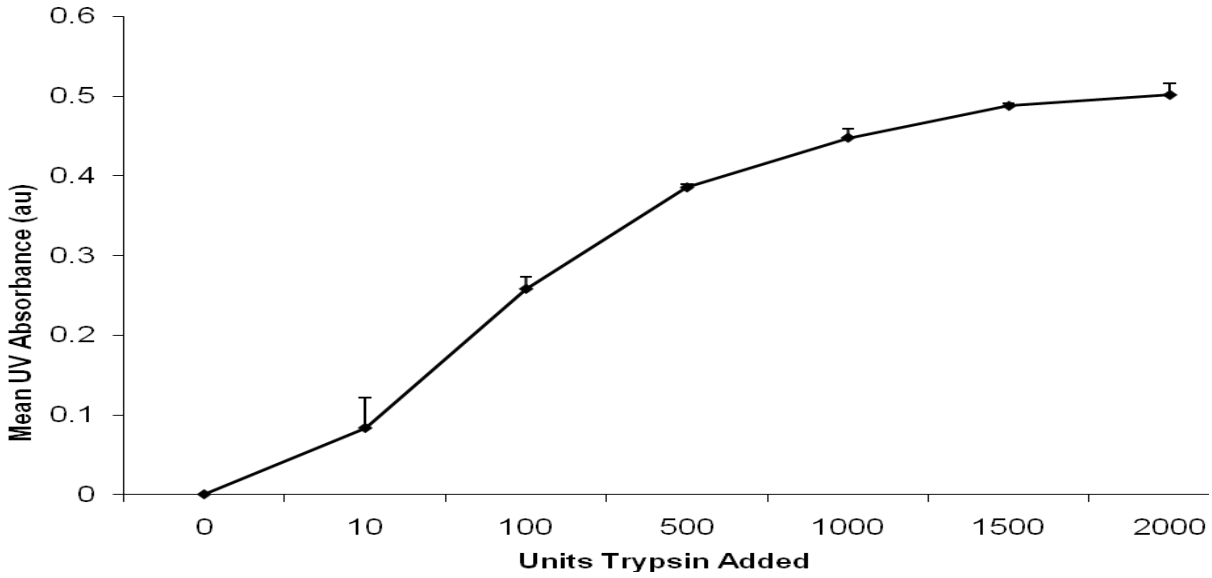


Figure 3.1: azocasein trypsin calibration. A range of trypsin units (10 – 2000) were assayed at pH 8 using the azocasein protease assay. Error bars represent standard deviation, n = 3.

Calibration was initially carried out using a range of between 10 to 2000 units of trypsin (Fig. 3.1). However, the assay began to plateau at approximately 1000 units of trypsin indicating that all the substrate that could be digested by trypsin had been digested. Thus a further calibration was carried out (again in triplicate), but this time using a serial dilution representing the steepest slope of the calibration curve, that is 0 to 100 units of trypsin (Fig. 3.2).

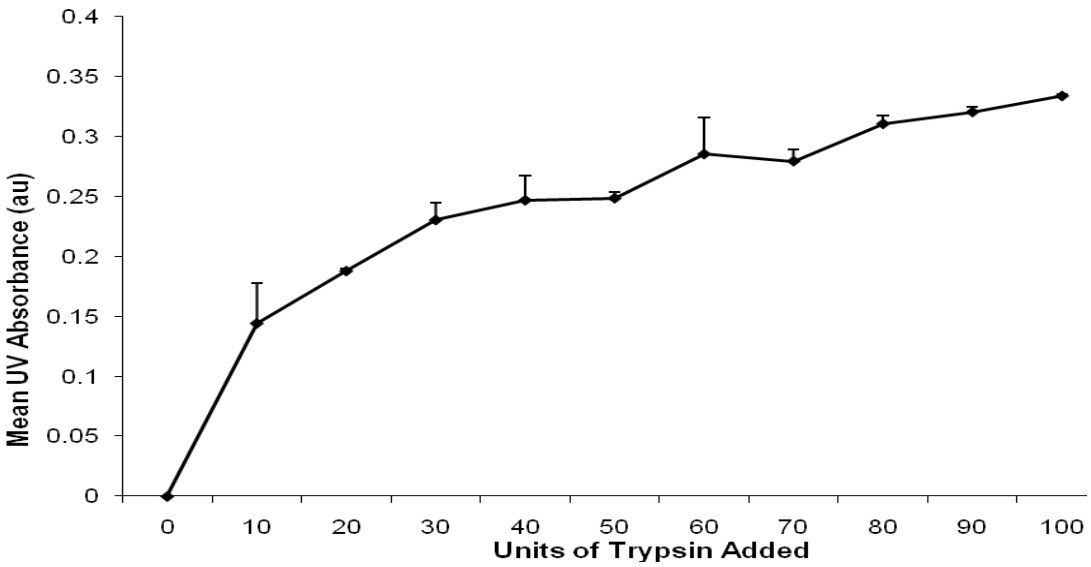


Figure 3.2: azocasein trypsin calibration. A range of 10 – 100 units of trypsin were assayed at pH 8 using the azocasein protease assay. Error bars represent standard deviation, n = 3.

3.2: Protease Activator and Inhibitor Studies on Malt

The contributions of the members of each of the four protease classes to the total levels of protease activity in days two and four barley malts was investigated using class specific protease inhibitors and the reducing agent DTT. DTT was included in all the activator and inhibitor study assays, but not in subsequent assays unless stated.

3.2.1: DTT

DTT is a reducing agent which activates cysteine proteases via the net reduction of their thiol groups. Studies have shown that the addition of DTT to green malt crude extracts and also mash samples increases the overall levels of protease activity (Jones & Budde, 2003) indicating a significant role for the cysteine class proteases in barley grain proteolysis. With this in mind, the proteolytic activity of two and four day germinated green malt crude extracts in the presence of 5 mM DTT, at different pH levels was determined (Figs. 3.3 and 3.4).

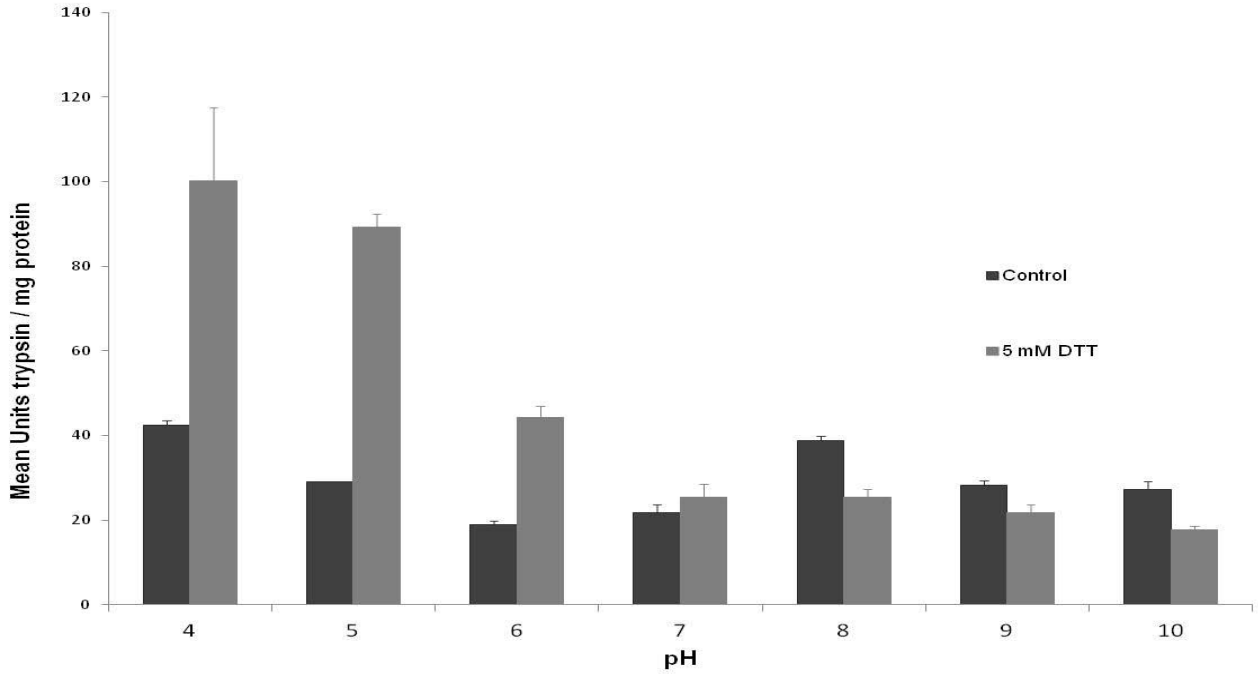


Figure 3.3: effects of 5 mM DTT on the protease activity of two day green malt crude extract. Addition of 5 mM DTT resulted in an increase in the levels of proteolytic activity at the lower end of the pH scale only, with a small decrease in protease activity observed above pH seven. Error bars represent standard deviation, n = 3.

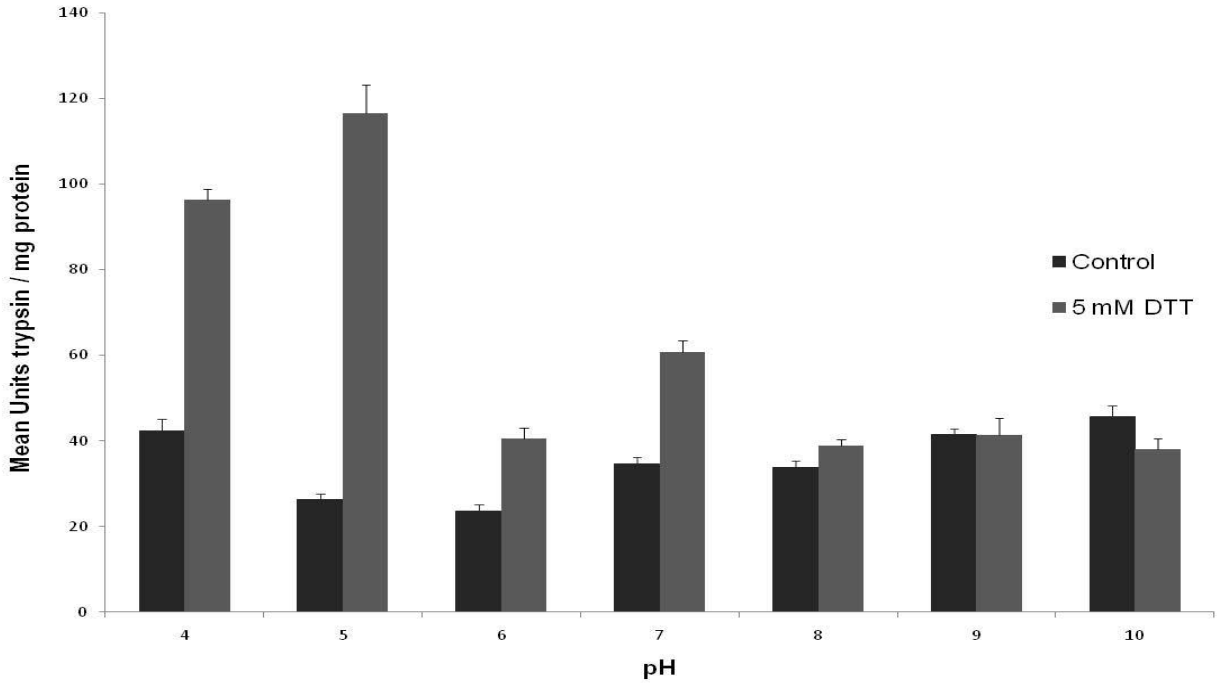


Figure 3.4: effects of 5 mM DTT on the protease activity of four day green malt crude extract. The addition of 5 mM DTT increases the levels of protease activity in four day green malt extract at the lower end of the pH scale investigated. Error bars represent standard deviation, n = 3.

This study showed that the addition of 5 mM DTT to both two and four day germinated green malt crude extracts greatly increased the levels of proteolytic activity at pH four to six, and that by day four the activity of mid range (pH seven) barley cysteine proteases also increased (Figs. 3.3 and 3.4). Furthermore, by comparing the two graphs (Figs. 3.3 and 3.4) it can be seen that the activity of non – cysteine class proteases is also higher in day four green malt than in day two at alkaline pHs, but that reducing conditions appear to slightly inhibit proteolysis at these pHs at two days of malting.

3.2.2: E – 64 Inhibition

In order to more thoroughly investigate the role of barley cysteine class proteases during the malting process the cysteine protease class specific inhibitor, E – 64 was incorporated into the assays. As with all the inhibitor studies in this section DTT was added to both control and crude extract assays.

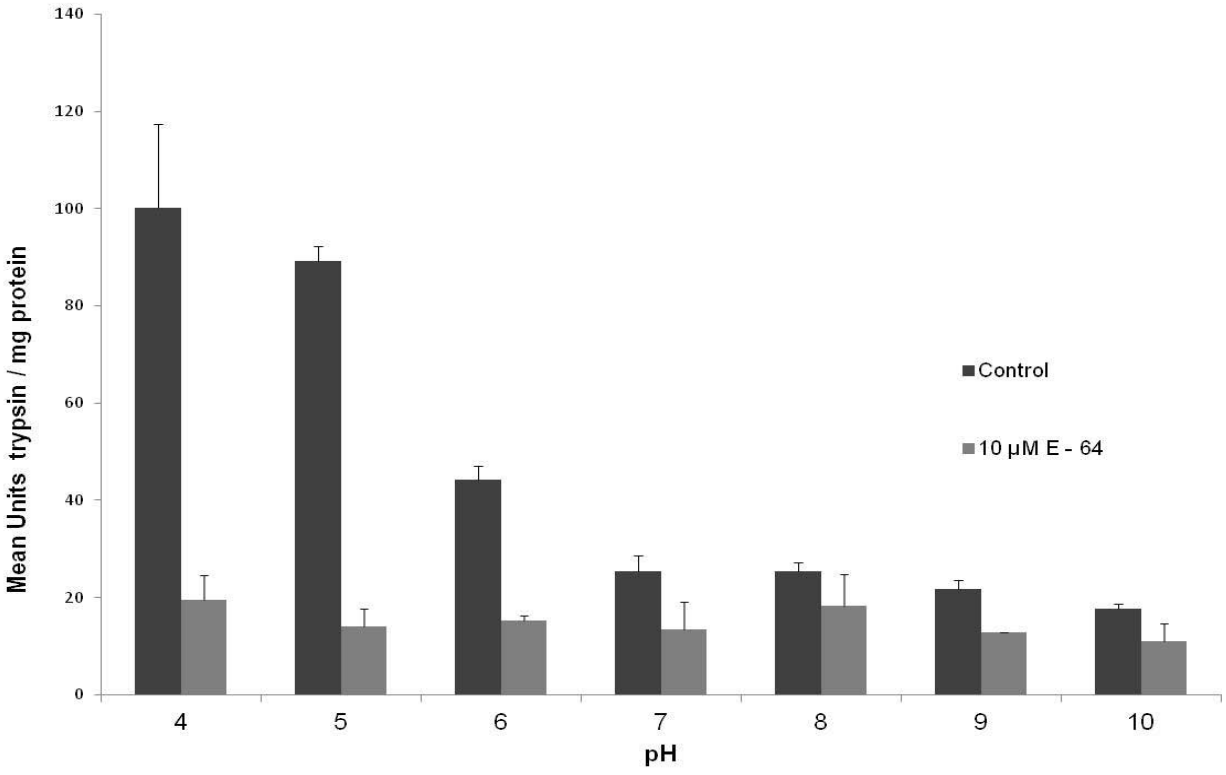


Figure 3.5: effects of 10 µM E – 64 on the protease activity of two day green malt crude extract. The addition of 10 µM E – 64 decreased the levels of protease activity at pH four and five, and less so at pHs six to ten. Error bars represent standard deviation, n = 3.

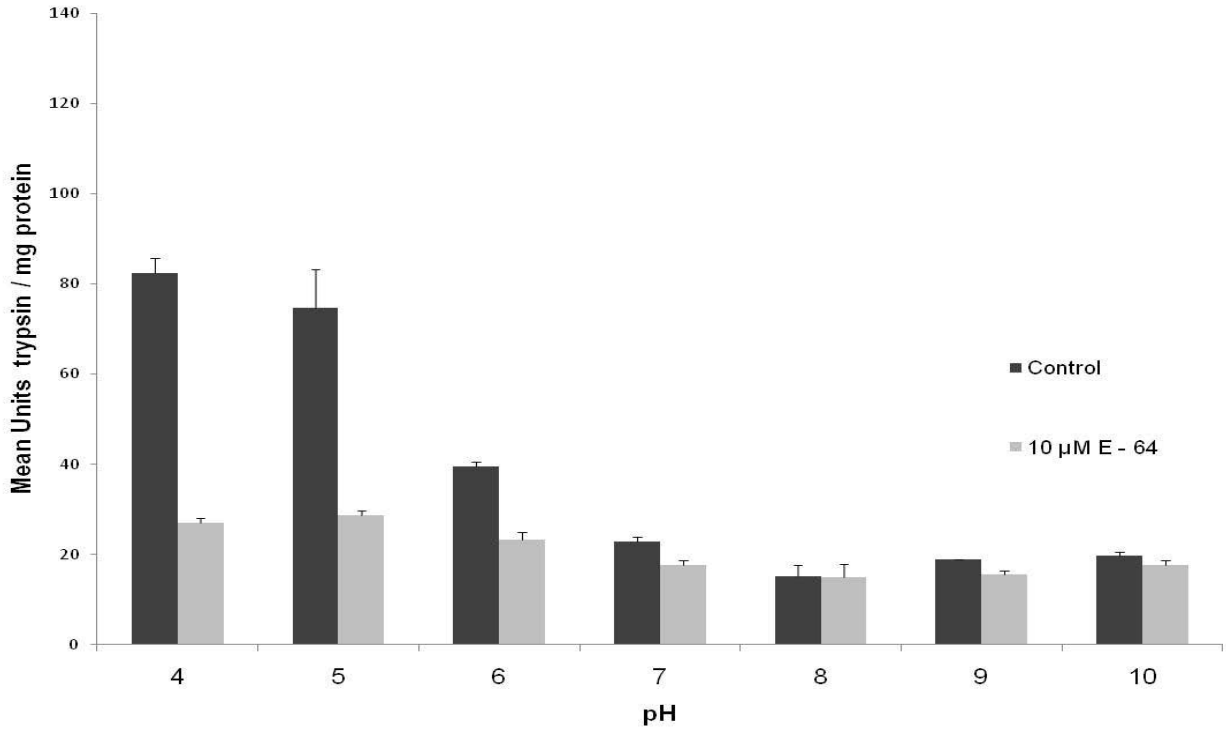


Figure 3.6: effects of 10 µM E – 64 on the protease activity of four day green malt crude extract. The addition of 10 µM E – 64 produced a decrease in the levels of protease activity at pH four to seven, with little difference in protease activity above pH seven. Error bars represent standard deviation, n = 3.

Figs. 3.5 and 3.6 show clearly a significant role for cysteine proteases in proteolysis at acidic pH, and show that it is mainly non – cysteine proteases which are active at alkaline pHs. This study also shows that the cysteine proteases make a larger contribution the overall levels of protease activity at day two of malting than at day four as the levels of inhibition achieved by the addition of 10 µM E – 64 is greater at day two than at day four (approximately 80 % inhibition at pH four and five in day two green malt compared to only about 60 % inhibition at the same pHs in day four green malt) (Figs. 3.5 and 3.6).

3.2.3: 1, 10 Phenanthroline Inhibition

Metalloprotease activity was selectively inhibited through the use of 1, 10 phenanthroline, a chelator of divalent cations with a strong affinity for zinc. A comparison of the data in Figs. 3.7 and 3.8 indicates that 1, 10 phenanthroline exerts its greatest inhibitory effect at the lower end of the pH range at day two (inhibition of approximately 50 % of the proteolytic

activity at pH four and five (Fig. 3.7)), indicating an acidic pH optimum for day two green malt metalloproteases. The overall contribution of metalloproteases is reduced by day four (Fig. 3.8), but at this time they still have an acidic pH optima. This data suggests that, just like the cysteine proteases, there is a stronger role for barley metalloproteases at day two of malting than at day four.

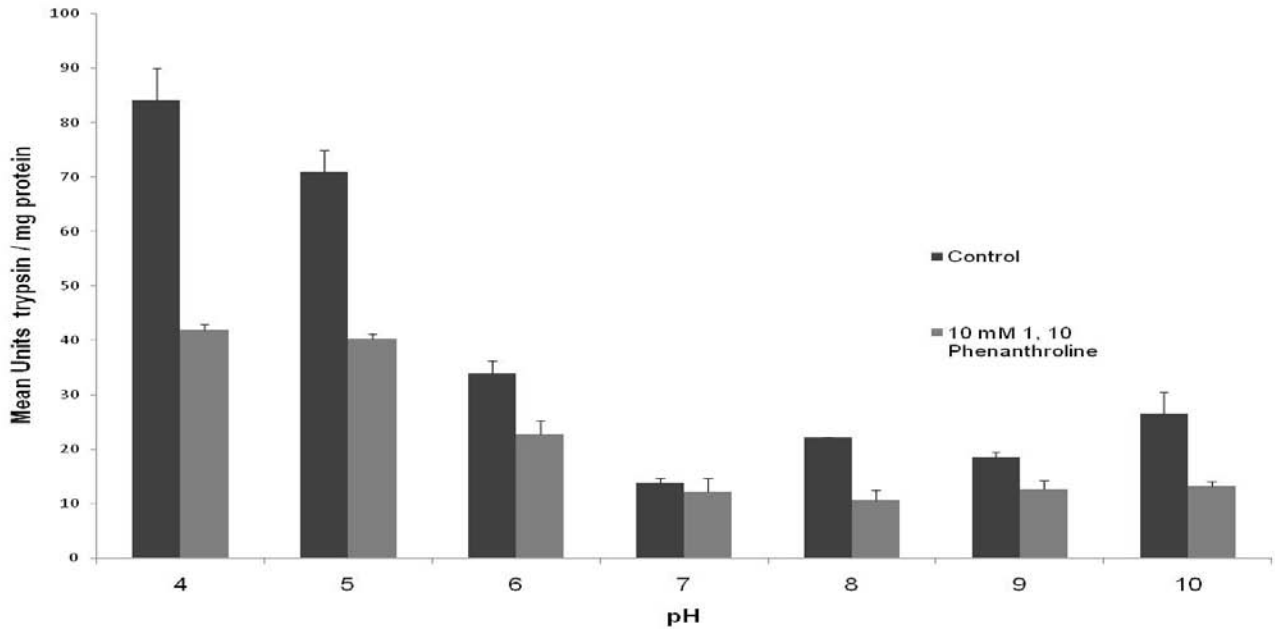


Figure 3.7: effects of 10 mM 1, 10 phenanthroline on the protease activity of two day green malt crude extract. The addition of 10 mM 1, 10 phenanthroline results in the inhibition of protease activity across the pH range of the study. Error bars indicate standard deviation, n = 3.

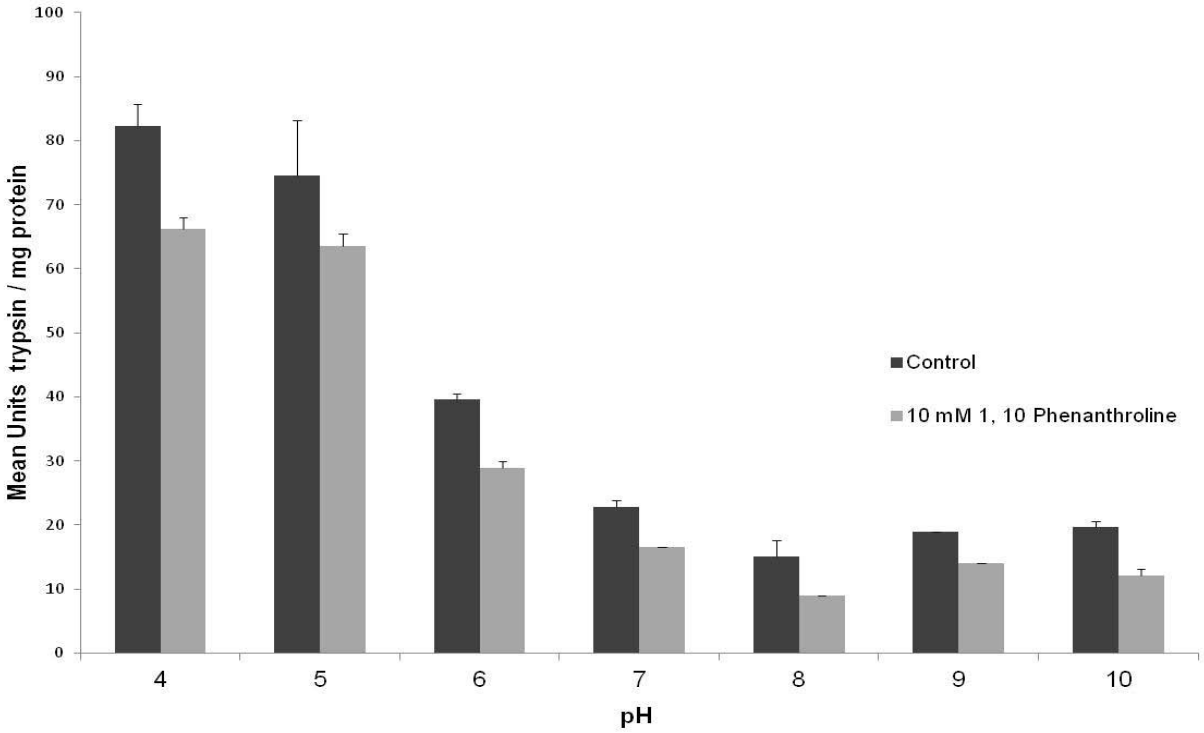


Figure 3.8: effects of 10 mM 1, 10 phenanthroline on the protease activity of four day green malt crude extract. The addition of 10 mM 1, 10 phenanthroline inhibited protease activity across the pH spectrum of this study. Error bars indicate standard deviation, n = 3.

3.2.4: Pepstatin A Inhibition

The inhibition of the aspartate class proteases was effected by the addition of 20 µM pepstatin A to the protease activity assays of both two and four day green malt. The results of these studies showed that just like the cysteine and metalloproteases, aspartate class barley grain proteases are primarily active at lower pHs (Figs. 3.9 and 3.10). The study also showed that the aspartate class proteases are active across the pH range of the study at day two of malting, but less so at day four. Furthermore, the day two aspartic class proteases had a higher specific activity (observed by the greater levels of inhibition of protease activity achieved by pepstatin A inclusion) than the day four aspartate proteases (Figs. 3.9 and 3.10), with the greatest difference in inhibition levels being observed at pH seven with approximately 66 % inhibition of protease activity at day two compared to about 20 % inhibition at pH seven at day four of malting.

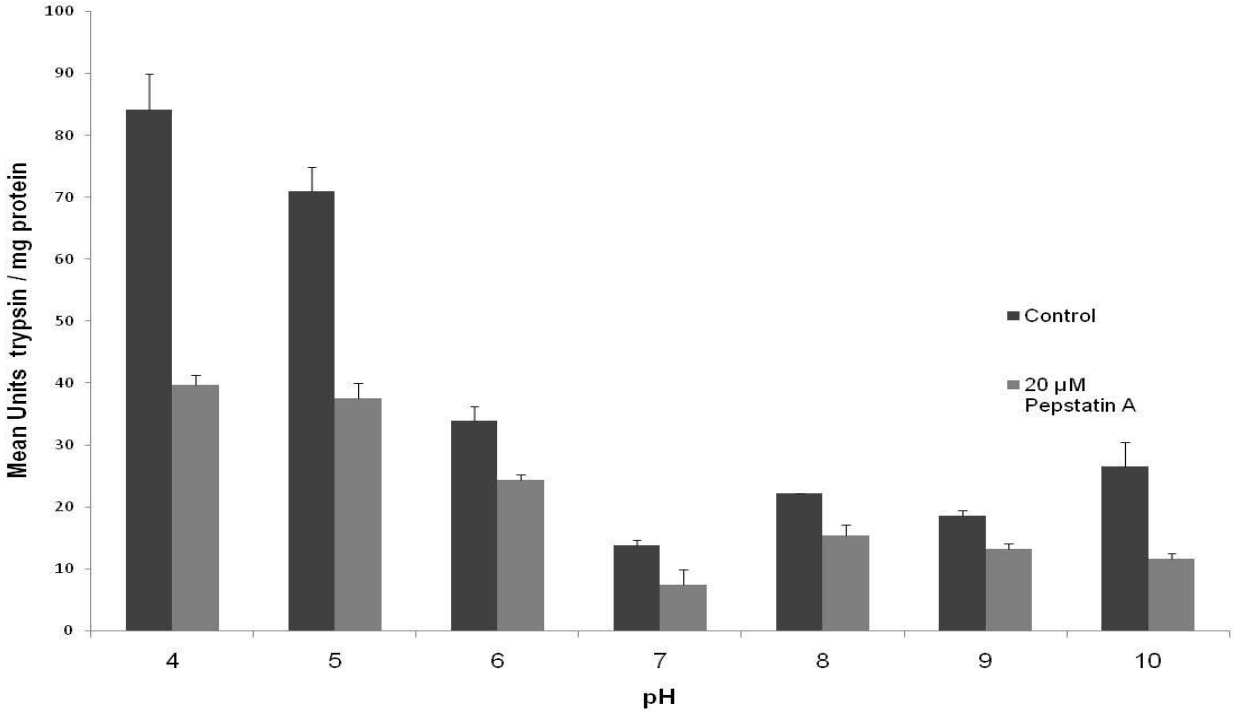


Figure 3.9: Effects of 20 µM pepstatin A on the protease activity of two day green malt crude extract. In day two green malt the aspartate class proteases had a pH optima lying between pH four and seven. Error bars indicate standard deviation, n = 3.

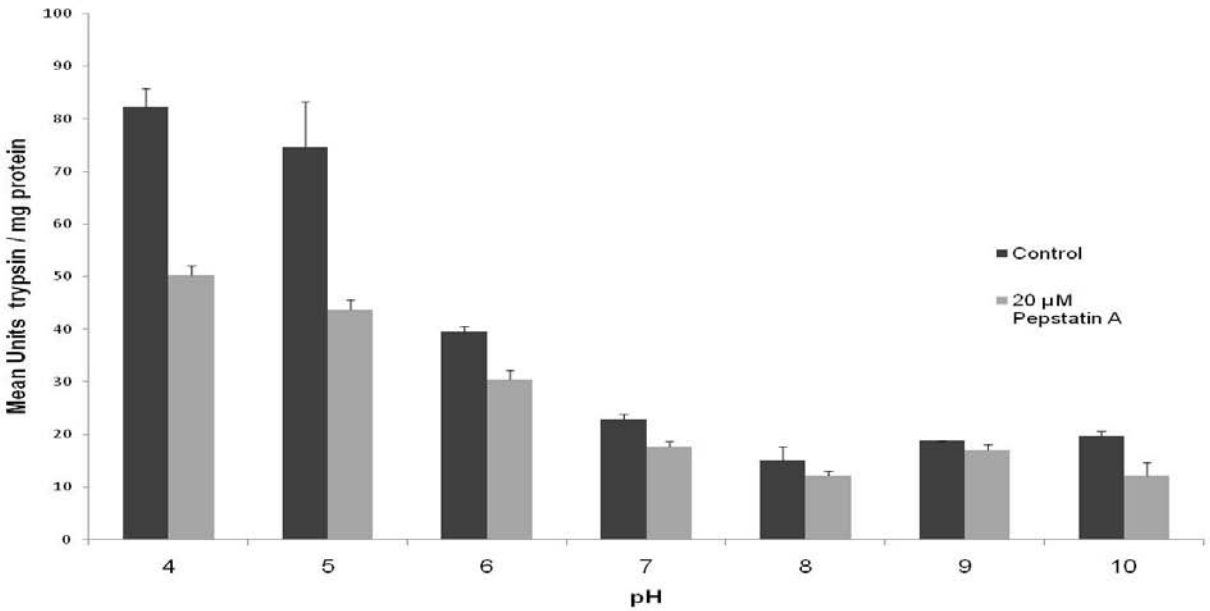


Figure 3.10: Effects of 20 µM pepstatin A on the protease activity of four day green malt crude extract. The aspartate proteases of four day green malt are active across the pH spectra and have an optimum pH of approximately four to five. Error bars represent standard deviation, n = 3.

3.2.5: PMSF Inhibition

PMSF is a selective inhibitor of serine and some cysteine class proteases. When 10 mM PMSF was included in the protease activity assays of both two and four day green malts inhibition was observed across the pH range in the day two malt but not in the day four malt (Figs. 3.11 and 3.12). The data also indicates a larger contribution for serine proteases to the overall levels of protease enzyme activity during the earlier stages of malting (approximately 69 % inhibition at pH four of day two green malt assays, compared to approximately 48 % inhibition at pH four in day four assays).

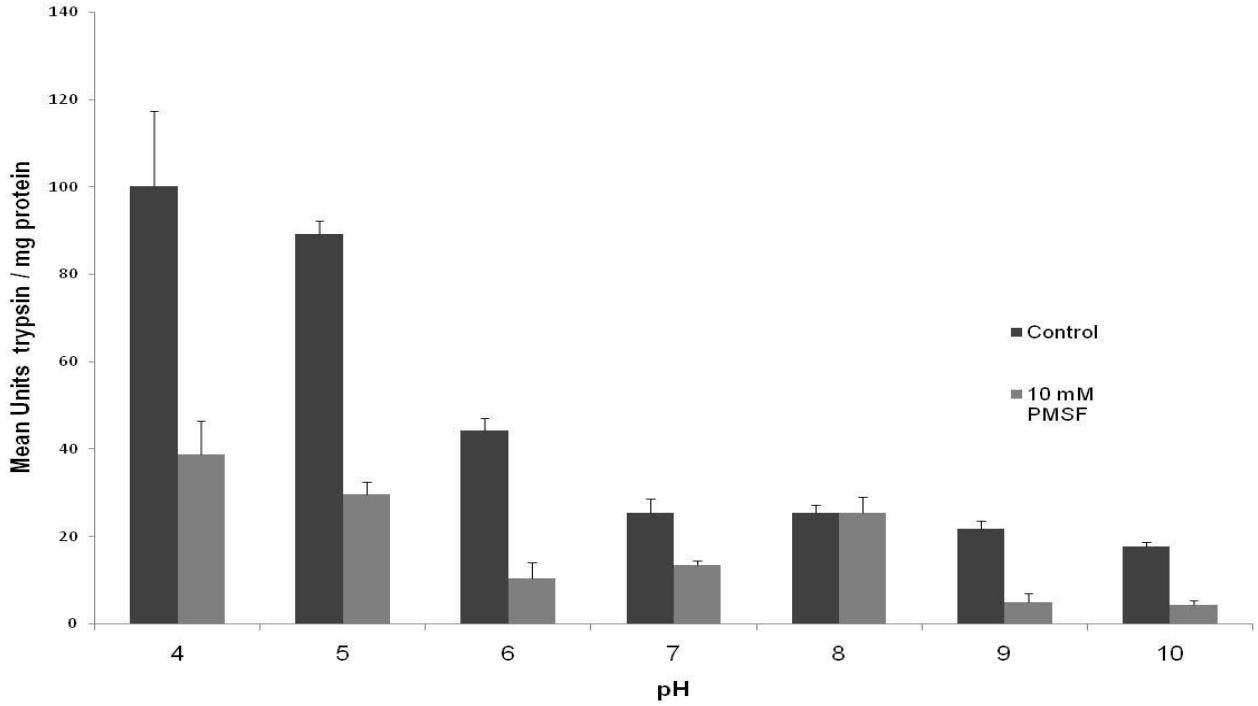


Figure 3.11: Effects of 10 mM PMSF on the protease activity of two day green malt crude extract. Serine proteases, at day two of malting, have a broad pH range of activity. Error bars represent standard deviation, n = 3.

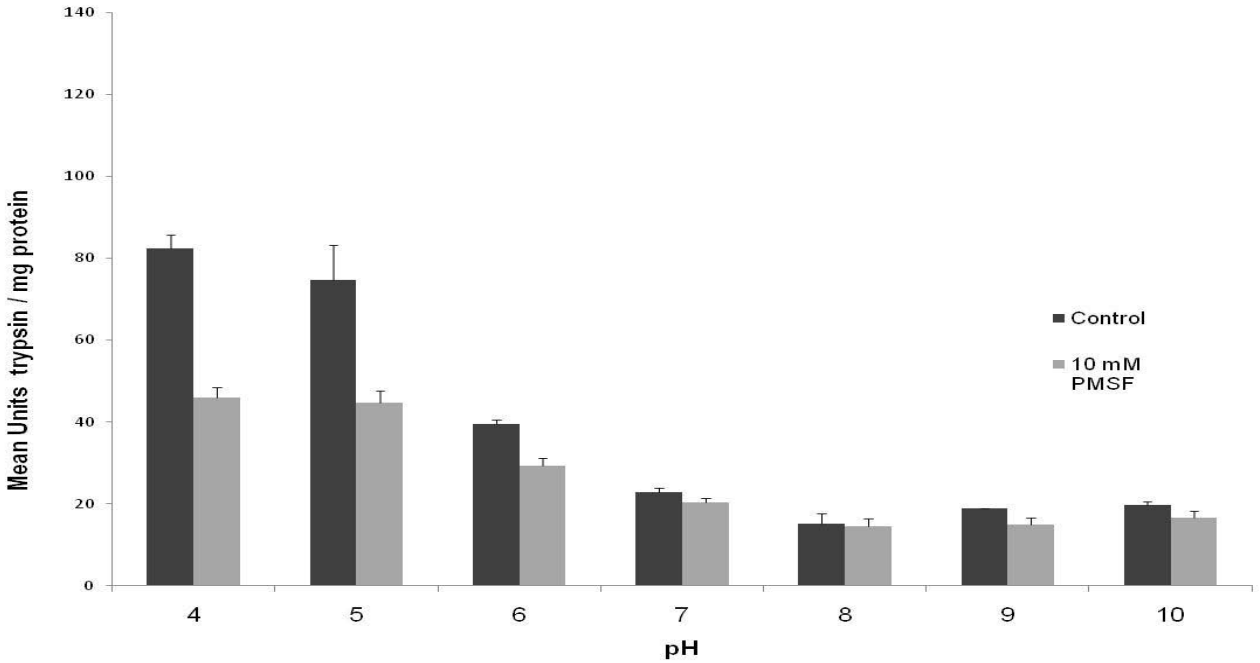


Figure 3.12: Effects of 10 mM PMSF on the protease activity of four day green malt crude extract. By day four of malting the pH range of serine proteases is limited to more acidic pH with their pH optima being between pH four and five. Error bars represent standard deviation, n = 3.

It should be noted however, that the control values are lower at low pH in the DTT studies (Figs. 3.3 and 3.4) than in any of the inhibitor studies which may indicate a problem with the experimental technique during the DTT studies such as pipetting errors, or that perhaps the stability of the proteolytic inhibitors or the protease / inhibitor complexes are affected by repeated green malt refreezing and prolonged storage at – 20^{o}C as the DTT studies were conducted first. Thus the absolute values for these studies cannot be directly compared but the general patterns of activation and inhibition are still comparable.

3.3: Metalloprotease Enrichment

The metalloproteases present in four day kilned malt crude extracts were further investigated using FPLC mediated fractionation of four day kilned malt crude extracts. The enrichment process consisted of three stages and involved three separate FPLC columns, azocasein protease activity assays of the initial crude extract and of selected fractions from each round of FPLC fractionation (assays were carried out with and without the class specific

metalloprotease inhibitor 1, 10 phenanthroline), and SDS – PAGE gel analysis of these fractions (see Fig. 2.2 for a schematic).

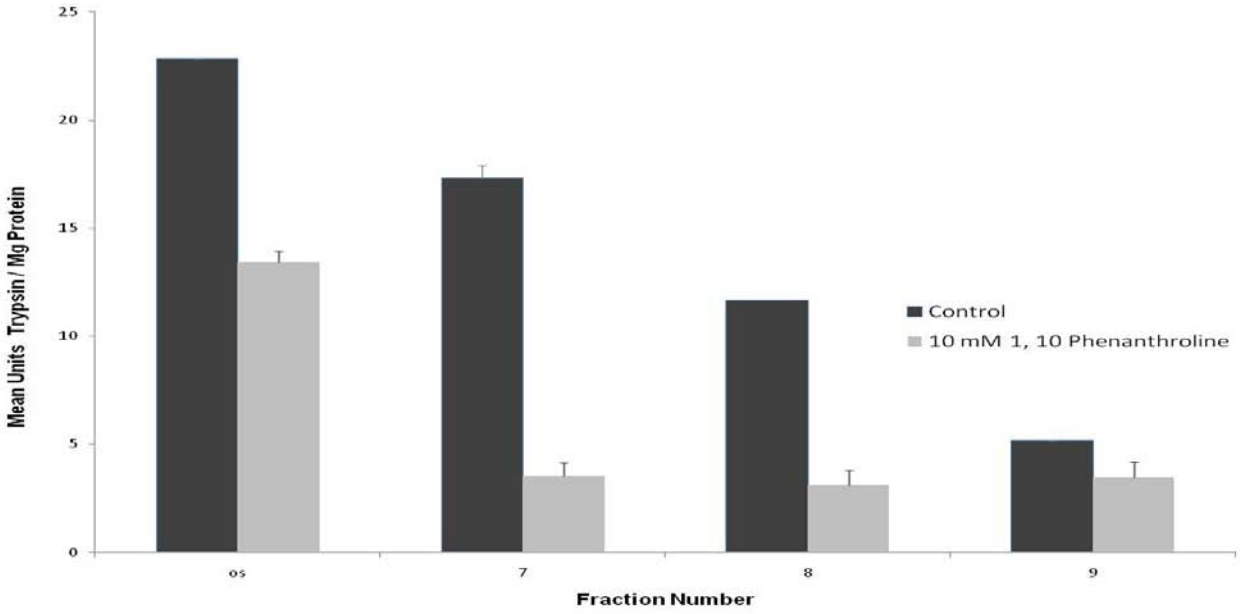


Figure 3.13: Effects of 10 mM 1, 10 phenanthroline on the protease activity of QFF anion exchange fractions from four day kilned malt. Protease activity assays carried out on FPLC fractions from the first stage of the enrichment process. Those which exhibited metalloprotease activity are shown above. "OS" represents the activity of a sample of the original four day malt crude extract. Error bars represent standard deviation, n = 3.

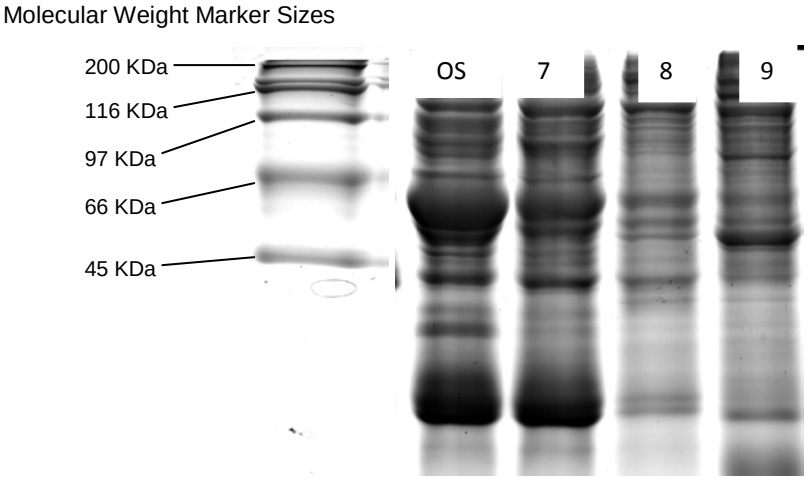


Figure 3.14: SDS PAGE analysis of FPLC fractions from the first stage (QFF anion exchange column) of the metalloprotease fractionation process. "OS" refers to the of four day kilned malt crude extract. The numbers refer to the fractions from the FPLC which exhibited metalloprotease activity.

The protease activity assays carried out on the FPLC fractions from the first stage of the enrichment process showed an increase in the proportion of protease activity that could be inhibited by 1, 10 phenanthroline in the active fractions compared to the original sample, which is indicative of metalloprotease enrichment in these fractions (Fig. 3.13). In the "OS" approximately 41 % of the total protease activity was inhibited by 1, 10 phenanthroline (Fig. 3.13) compared to 78 % and 70 % in fractions seven and eight respectively. SDS – PAGE analysis of these fractions and the "OS" sample showed differing polypeptide profiles between the samples (Fig. 3.14) indicating that fractionation of the four day malt crude extract was occurring, but there were still too many proteins present in the fractions for mass spectroscopy analysis.

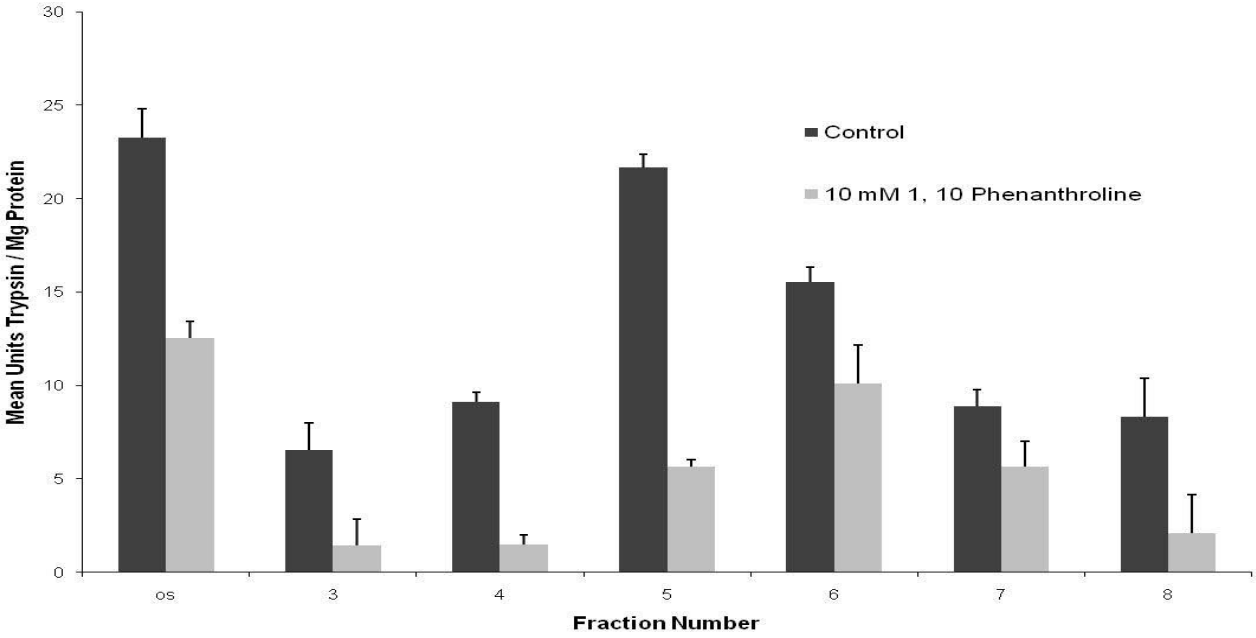


Figure 3.15: Effects of 10 mM 1, 10 phenanthroline on the protease activity of gel filtration fractions from the second stage of the metalloprotease enrichment process. "OS" represents a sample of the pooled fractions from stage one of the enrichment process. Errors bars indicate standard deviation, n = 3.

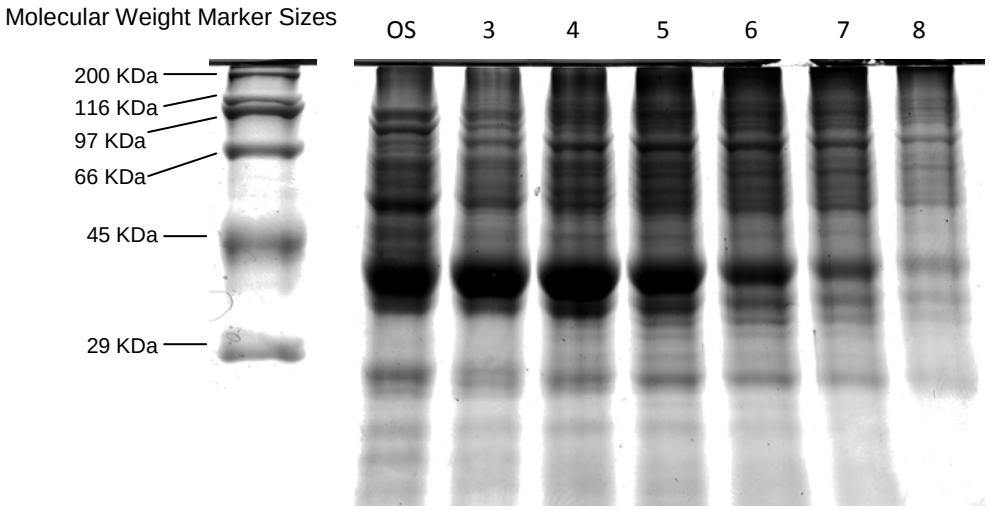


Figure 3.16: SDS - PAGE analysis of the metalloprotease activity containing fractions from the gel filtration stage of the enrichment process. "OS" represents the pooled metalloprotease containing fractions from the first stage of enrichment, and the numbers refer to the FPLC fraction that the samples originated from.

The second stage of the fractionation process used a Superose 12 gel filtration column and the protease activity assays on the eluted fractions indicated further enrichment of metalloprotease activity. When the active metalloprotease fractions were analysed by SDS – PAGE it was again observed that too many proteins were present for mass spectroscopy analysis. Thus the high activity metalloprotease containing fractions (fractions 3 to 5) were pooled and put through a further fractionation step (Fig. 2.2). It can be observed in Fig. 3.16 that despite gel filtration mediated separation of the pooled fractions, there were still a high number of low molecular weight proteins present in the gel filtration fractions investigated. This was unexpected as gel filtration columns separate proteins based upon molecular size with the largest proteins eluting first, thus small molecular weight proteins (such as those with MW below 29 KDa) would be expected to elute later than larger proteins, thus their presence in the fractions investigated was unexpected. However, this unexpected observation could be due to the formation of aggregates within the crude extract or the pooled fractions, which are not broken up until separation on the SDS – PAGE gel.

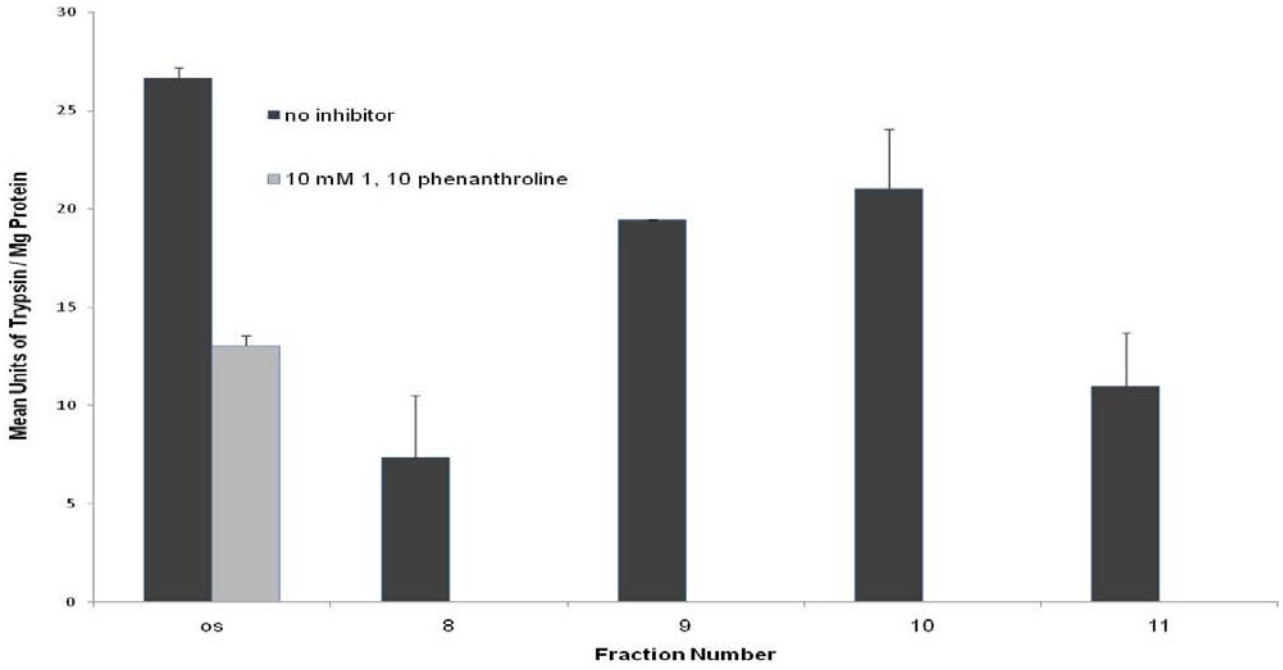


Figure 3.17: Effect of 10 mM 1, 10 phenanthroline on the protease activity of fractions from Mono Q anion exchange fractionation. "OS" represents the pooled fractions from gel filtration fractionation. Errors bars indicate standard deviation, n = 3.

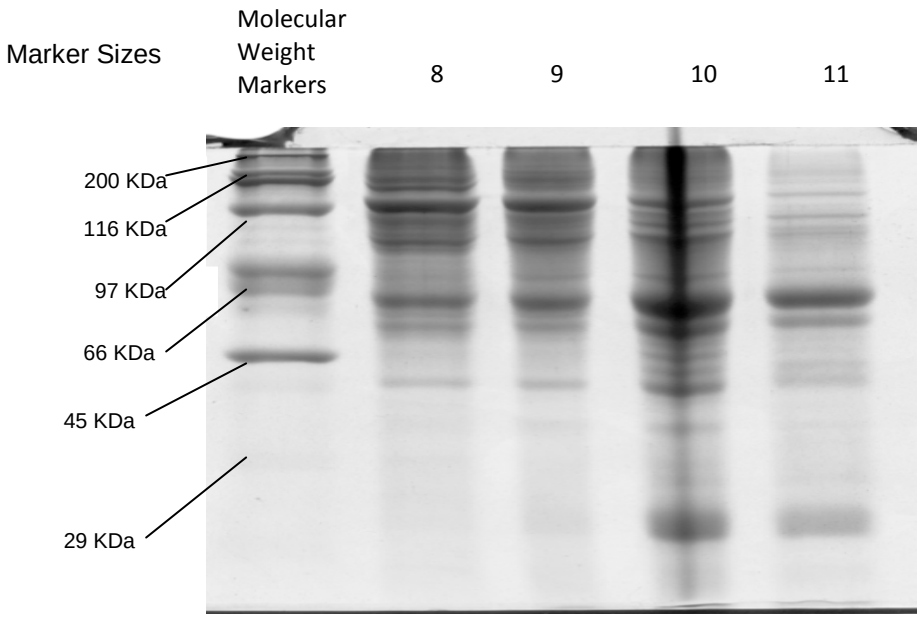


Figure 3.18: SDS – PAGE analysis of metalloprotease activity containing fractions from Mono Q anion exchange chromatography. Numbers correspond to the fractions from the final stage of the metalloprotease enrichment process.

By the third round of fractionation all the FPLC fractions containing protease activity had their activities completely inhibited by the addition of 10 mM 1, 10 phenanthroline (Fig. 3.17) indicating that the protease activity in these fractions consisted of only metalloproteases. However, these fractions also contained a large number of other proteins (Fig.3.18) thereby rendering any protease identification by mass spectroscopy difficult. Thus a modified enrichment procedure was attempted incorporating an ammonium sulphate precipitation step (see sections 2.4.3 & 2.4.4).

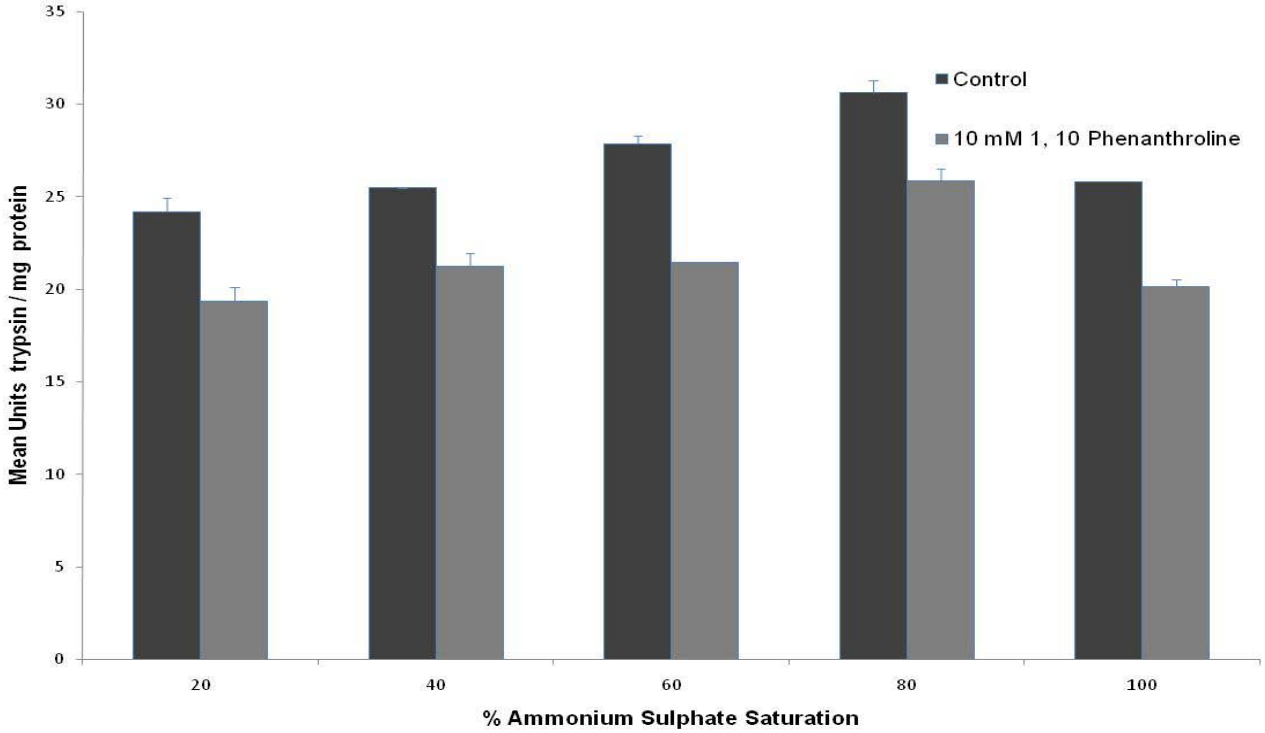


Figure 3.19: Metalloprotease activity present in different ammonium sulphate fractions of an extract prepared from four day kilned malt. Different levels of ammonium sulphate saturation were investigated for their metalloprotease activity in the precipitate. All fractions exhibited metalloprotease activity with the 80 % fraction showing slightly higher levels. Error bars show standard deviation, n = 3.

The precipitate from every ammonium sulphate fraction contained some level of protease activity which could be inhibited by 1, 10 phenanthroline (Fig. 3.19), with the 80 % fraction containing the most protease activity. The 80 % fraction was thus selected and, in order to remove the ammonium sulphate, was put through a desalting column before being loaded

onto the QFF anion exchange column for the first round of metalloprotease fractionation. Fig. 3.20 shows the results of azocasein endoprotease activity assays carried out on the 80 % ammonium sulphate fraction before it was applied to the desalting column and the pooled protein containing desalting column fractions. No data is presented from the QFF anion exchanger column as none of the resulting fractions exhibited any protease activity.

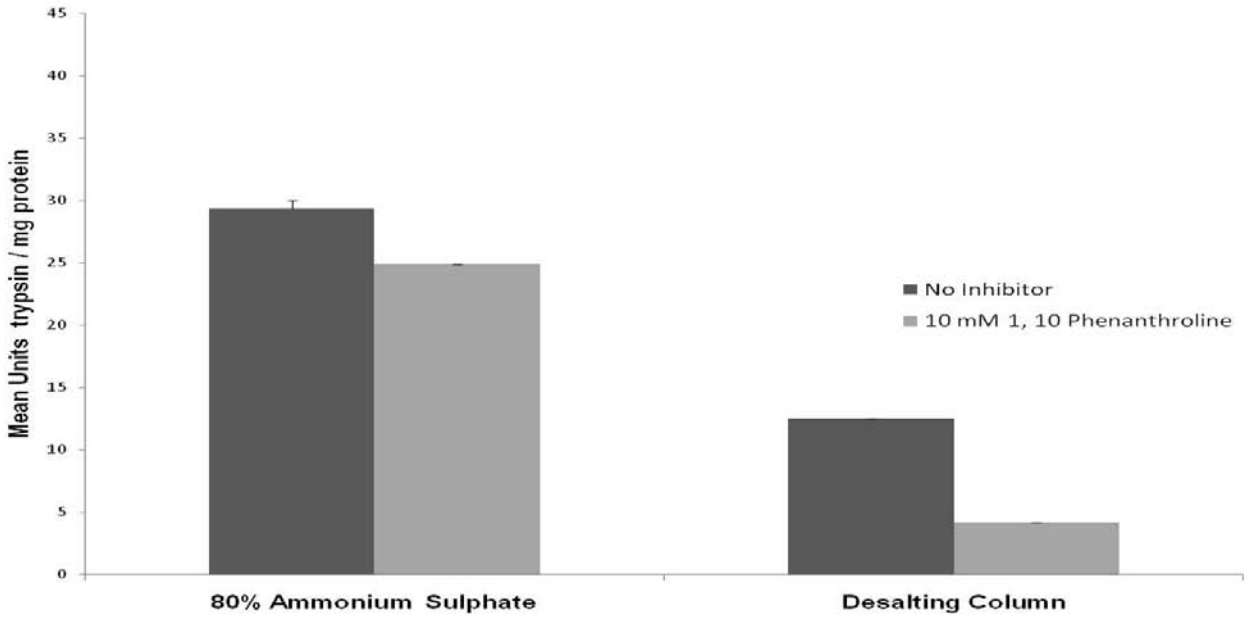


Figure 3.20: Comparison of the metalloprotease activities present in fractions from 80 % ammonium sulphate precipitation and from the desalting column. The overall levels of protease activity in the combined fractions from the desalting column is approximately 50 % lower than that of the original ammonium sulphate fraction, but that a greater proportion of this activity is metalloprotease due to the almost 70 % inhibition achieved by the application of 1, 10 phenanthroline.

The protease activity in the desalting column pooled samples was approximately 50 % lower than in the 80 % ammonium sulphate fraction (Fig. 3.20), but the proportion of activity that could be inhibited by 10 mM 1, 10 phenanthroline was larger (approximately 66 % compared to 16 % in the 80 % ammonium sulphate fraction) this enhancement of metalloprotease activity in the desalting column fractions, could possibly be the result of the removal of small metalloprotease inhibitor molecules by the desalting column, which might also include ammonium sulphate.

The reduction in the overall levels of protease activity observed in the pooled fractions from the desalting column (Fig 3.20) combined with the lack of any protease activity observed in the QFF column fractions highlights an important problem associated with the purification of proteases in general, that they are by their nature inherently unstable and thus can be difficult to purify.

3.4: The Effects of Class Specific Protease Inhibitors on Grain Germination and the Activities of Starch Degrading Enzymes

3.4.1: Grain Germination and Protease Activity

During the germination studies described below, observations were made on the effects of the different class specific protease inhibitors on the germination process as a whole in order to investigate the importance of each of the protease class to this process (Figs. 3.21 – 3.24).

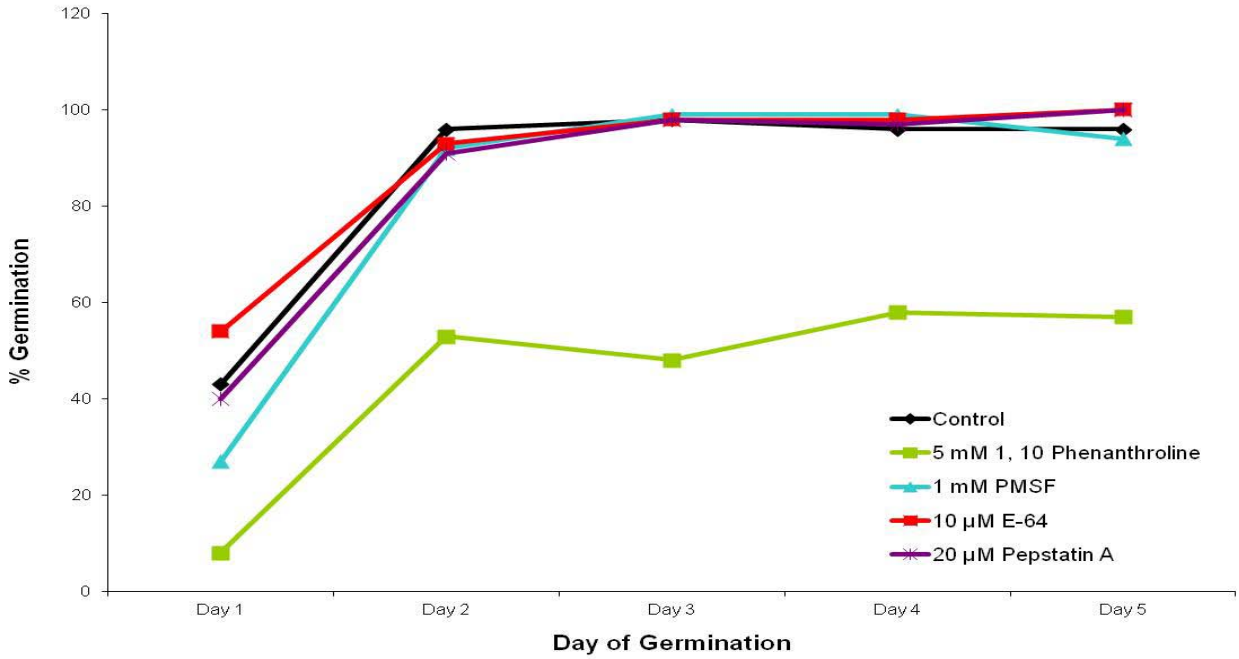


Figure 3.21: Effects of different class specific protease inhibitors on germination of barley grains. When different class specific protease inhibitors were included in the incubation media of 100 barley grains none of the inhibitors had any major effect on the levels of germination except for 1, 10 phenanthroline, a metalloprotease inhibitor, which inhibited germination.

Figs. 3.21 to 3.23 show that in the presence of 5 mM 1, 10 phenanthroline there is a marked decrease in the % germination, (as determined by grain chitting) (with just under 60 % of the grains germinated on day five compared to 100 % in the control grains (Fig. 3.21)), shoot emergence (less than 20 % of grains displaying shoots by day five of germination (Fig. 3.22) compared to 100 % by day five in the control grains) and average rootlet length (reaching a maximum of approximately 15 mm by day five (Fig. 3.23), whereas the control grains had had a maximum rootlet length of approximately 45 mm at day five of germination) indicating a possible role for the metalloproteases in the onset of barley grain germination and early plant growth. However, 1, 10 phenanthroline exerts its inhibitory effects through the chelation of divalent cations thus it could be that it is not the metalloproteases that are important for the onset of germination and early plant growth, but the presence of divalent cations within the grain (or specific grain compartments) that is important.

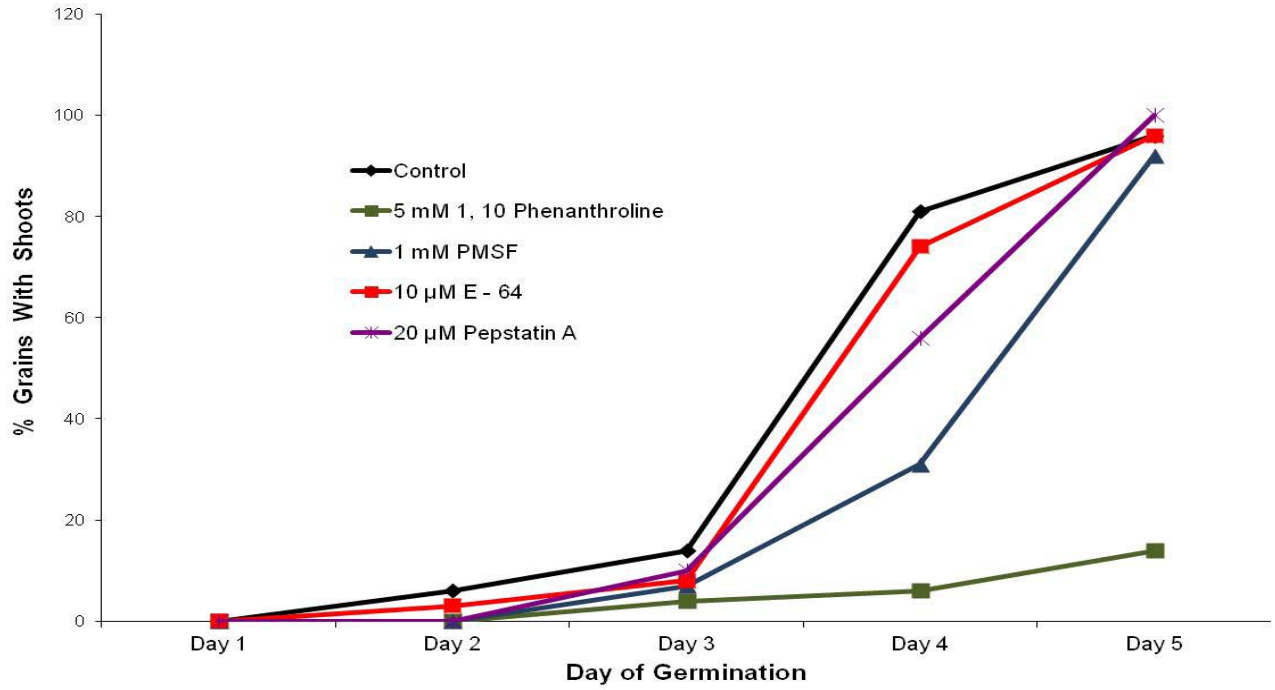


Figure 3.22: Effects of different class specific protease inhibitors on shoot emergence in barley grains. When 100 barley grains were germinated on the presence of different class specific inhibitors changes in shoot emergence were observed. Those grains germinated on the presence of 1, 10 phenanthroline (the class specific metalloprotease inhibitor) showed far lower levels of shoot development than any of the other grain studies. Grains germinated in the presence of PMSF (class specific serine protease inhibitor) and pepstatin A (class specific aspartate protease inhibitor) also showed a small decrease in shoot emergence levels

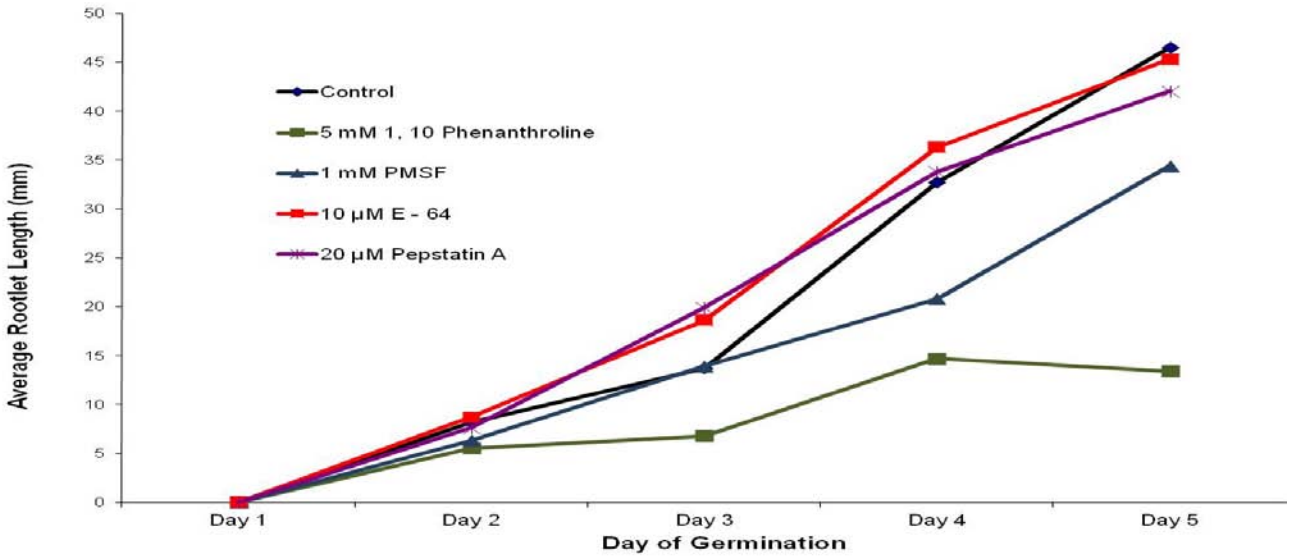


Figure 3.23: Effects of different class specific protease inhibitors on the average rootlet length in barley grains. The class specific protease inhibitors 1, 10 phenanthroline (class specific metalloprotease inhibitor) and PMSF (class specific serine protease inhibitor) had the greatest effects on average rootlet length especially at later days of germination.

The effects of the different class specific inhibitors, when included in the germination medium, on the overall levels of protease activity (measured at pH 5.0) at different days of germination was also investigated (Fig. 3.24). Two – way ANOVA and Bonferroni post – hoc analysis of the data showed that only the inclusion of 5 mM 1, 10 phenanthroline in the grain incubation media resulted in a significant ($p = < 0.05$) decrease in the overall levels of protease activity at all five days of germination (Fig. 3.24), suggesting that metalloproteases may be important in regulating overall protease levels during germination. However, 1, 10 phenanthroline is a divalent cation chelator, the 1, 10 phenanthroline treated grains showed inhibited germination (Fig. 3.21) and since barley grain proteases are thought to be synthesised during germination (Jones, 2005), the observed decrease in protease activity is probably more representative of a reduction in protease synthesis through an inhibition of the germination process rather than any major contribution of the metalloproteases. The only other inhibitors (Fig. 3.24) to produce a significant difference in the overall levels of protease activity on multiple days of germination was 1 mM PMSF at days one (where it increased the overall levels of protease activity) and day five (where it reduced the overall levels of protease activity). This result indicates that, at day five of germination, serine (and possibly some cysteine) proteases are an important component of the overall levels of

protease activity. The significant increase in the overall levels of protease activity observed at day one in the presence of 1 mM PMSF could show that serine proteases may be involved in the regulation of other protease classes, possibly by their degradation, thus inhibiting the serine proteases produces an increase in overall protease activity. The inclusion of 20 µM pepstatin – A into the barley grain germination media also resulted in a significant increase in the overall levels of protease activity at day one of germination. This could also indicate a regulatory role for the aspartate class proteases during early germination.

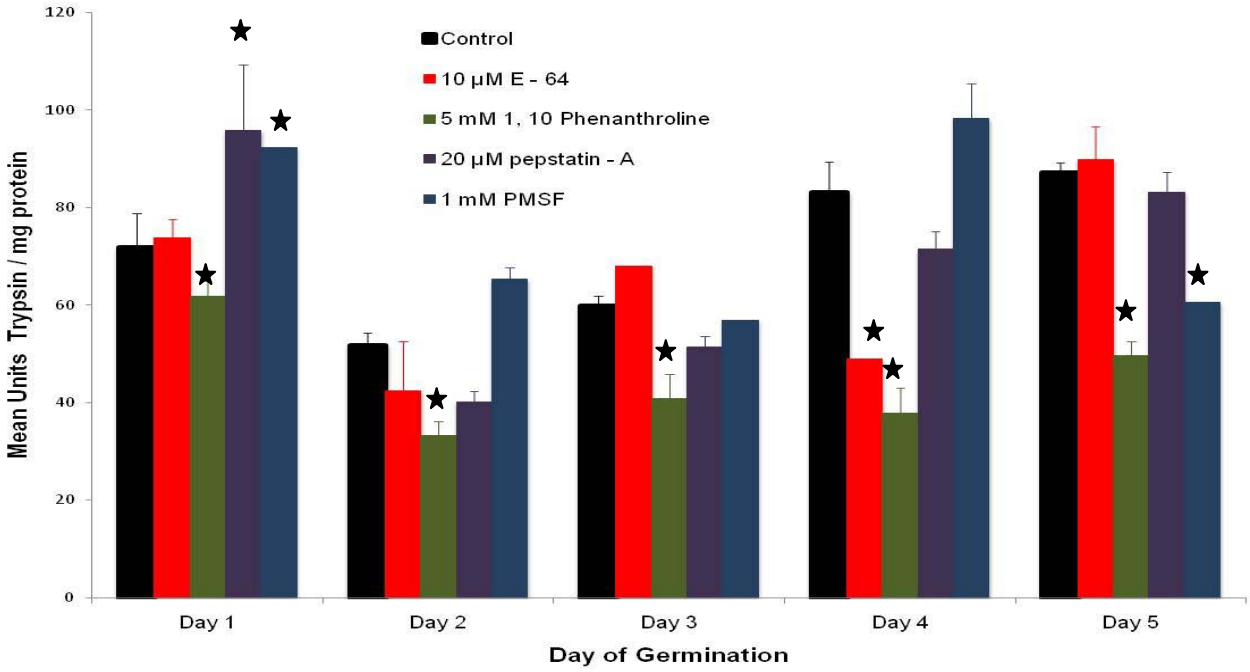


Figure 3.24: Effects of different class specific protease inhibitors on the overall levels of protease activity during germination. Protease activity assays were carried out at pH 5.0. The grains germinated in the presence of 5 mM 1, 10 phenanthroline showed a significant ($p = < 0.05$) decrease in protease activity. At day one of germination, those grains exposed to 1 mM PMSF showed a significant increase ($p = < 0.05$) in the overall levels of protease activity, as did the grains exposed to 20 µM pepstatin – A. Furthermore, at day four of germination the grains germinated in the presence of 10 µM E – 64 showed a significant decrease in the levels of protease activity. Error bars represent standard deviation, n = 3, ★ = Significant differences ($p = < 0.05$) in protease activity between the control and inhibitor on that particular day of germination

3.4.2: Divalent Cations and 1, 10 Phenanthroline

To investigate the potential importance of divalent cations in the germination process further germination studies with 5 mM 1, 10 phenanthroline were carried out, but this time incorporating different divalent cations into the germination media along with 1, 10 phenanthroline (Figs. 3.25 to 3.35).

When 1 mM, 5 mM and 10 mM $ZnSO_4$ were included along with 5 mM 1, 10 phenanthroline in the germination media of barley grains, a complete rescue in the overall levels of germination was observed with 5 mM $ZnSO_4$ and 10 mM $ZnSO_4$ only, with 1 mM $ZnSO_4$ only partially rescuing germination levels (Fig. 3.25).

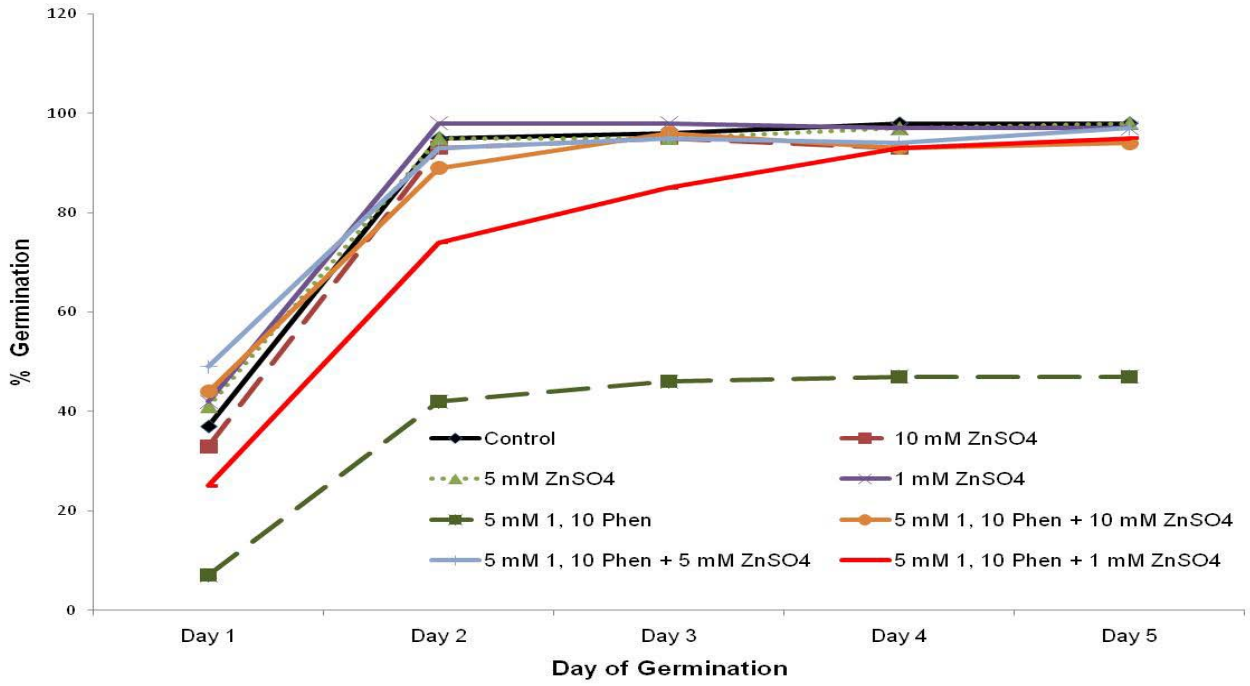


Figure 3.25: Effects of 5 mM 1, 10 phenanthroline and varying $ZnSO_4$ concentrations on the germination levels of barley grains. The inclusion of 5 mM and 10 mM $ZnSO_4$ with 5 mM 1, 10 phenanthroline in the germination media of barley grains rescued the 1, 10 phenanthroline mediated inhibition of germination to the control levels. The inclusion of 1 mM $ZnSO_4$ along with 5 mM 1, 10 phenanthroline also rescued germination levels but not to that seen in the control grains

The complete rescue of the 5 mM 1, 10 phenanthroline – mediated inhibition of germination by 5 mM and 10 mM $ZnSO_4$ indicates a stochiometric interaction between $ZnSO_4$ and 1, 10

phenanthroline as it was not until an equimolar $ZnSO_4$ concentration was added that full reversal of inhibition was observed (Fig. 3.25). When $ZnSO_4$ was added to the germination media in the absence of 5 mM 1, 10 phenanthroline little difference was observed in the levels of germination (Fig. 3.25) indicating that, at least in terms of germination levels, the grains could tolerate the presence of zinc. Germination physiology was affected however, as seen in observations concerning the levels of shoot emergence (Fig. 3.26). In these studies the inclusion of 10 mM $ZnSO_4$ (and to a smaller extent 5 mM $ZnSO_4$) to the germination media (in the absence of 5 mM 1, 10 phenanthroline) resulted in an inhibition of shoot emergence demonstrating that different components of the germination process may have differing sensitivities to the levels of zinc in their environment.

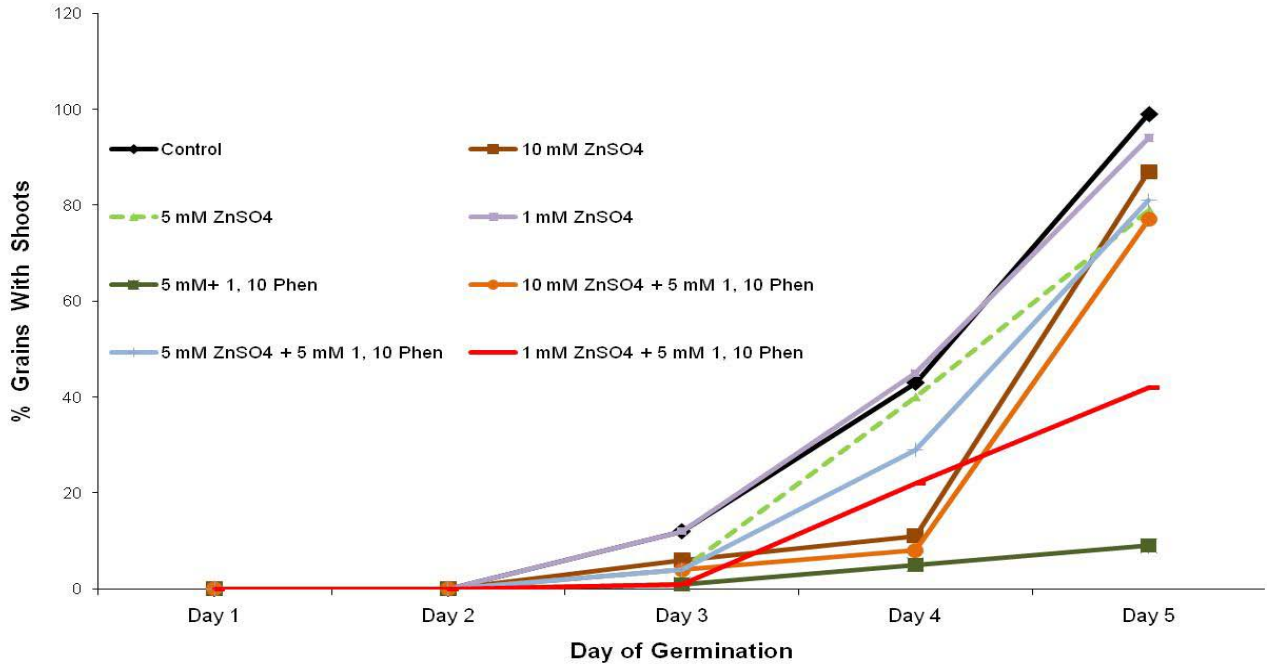


Figure 3.26: Effects of 5 mM 1, 10 phenanthroline and varying $ZnSO_4$ concentrations on shoot emergence in barley grains. None of the $ZnSO_4$ concentrations investigated resulted in a complete rescue of the 5 mM 1, 10 phenanthroline mediated inhibition of shoot emergence to the control levels, with 5 mM $ZnSO_4$ rescuing to the greatest extent. When $ZnSO_4$ was included alone in the germination media (i.e. in the absence of 5 mM 1, 10 phenanthroline) the 5 mM and 10 mM concentrations resulted in a decrease in the overall levels of shoot emergence.

Differences between the effects of the different zinc concentrations on the rescue of the 1, 10 phenanthroline inhibition of shoot emergence (Figs. 3.25 and 3.26) were also observed. All three $ZnSO_4$ concentrations resulted in partial rescue of the 1, 10 phenanthroline

mediated inhibition of shoot emergence (Fig. 3.26) but none resulted in total rescue. Furthermore, while 5 mM $ZnSO_4$ compensated shoot emergence to a greater extent than 1 mM $ZnSO_4$, the inclusion of 10 mM $ZnSO_4$ was less effective as shoot emergence was later (from day four rather than day three) and to a lesser extent than the two lower concentrations. This result could again be reflective of the zinc sensitivity of shoot emergence.

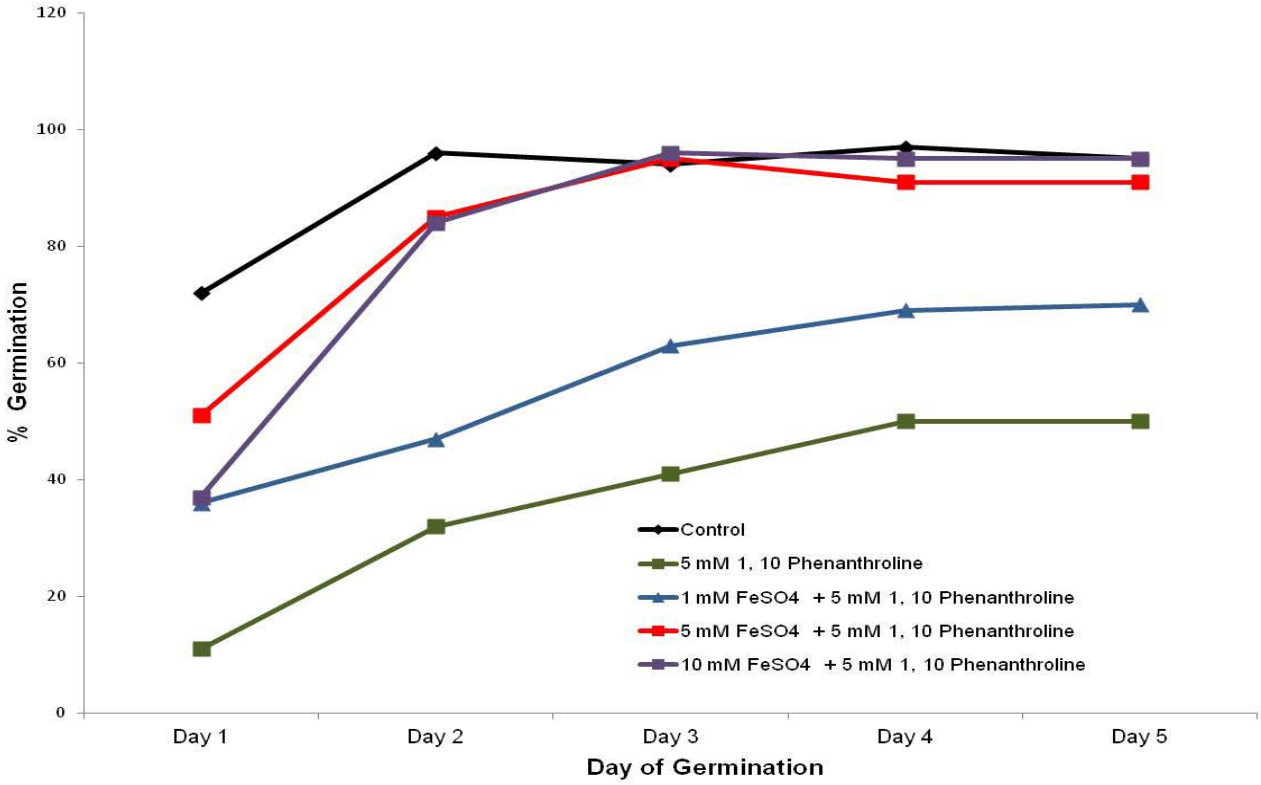


Figure 3.27: Effects of 5 mM 1, 10 phenanthroline and varying $FeSO_4$ concentrations on barley grain germination. The inclusion of 1 mM, 5 mM and 10 mM $FeSO_4$ into the germination media (along with 5 mM 1, 10 phenanthroline) resulted in the total rescue germination by day three with 5 mM and 10 mM $FeSO_4$, but only a partial rescue with 1 mM $FeSO_4$

When 1mM, 5 mM and 10 mM $FeSO_4$ was added alongside 5 mM 1, 10 phenanthroline increases in the germination levels back to those of the control levels were observed (Fig. 3.27). However, unlike $ZnSO_4$ which recovered the levels of germination from day one (Fig. 3.25), it was not until day three of germination that 5 mM and 10 mM $FeSO_4$ recovered germination to the level of the control grains. But, just like with 1mM $ZnSO_4$ the addition of 1 mM $FeSO_4$ only partially recovered germination (Fig. 3.27).

When 1mM, 5 mM and 10 mM $FeSO_4$ are added alone there was a slight decrease in the levels of germination observed during the first two days (Fig. 3.28), but inhibition was no longer present by day three of germination as there was no longer a difference between the $FeSO_4$ exposed and control grains. This observation points to a possible sensitivity to external iron in the early stages of barley grain germination.

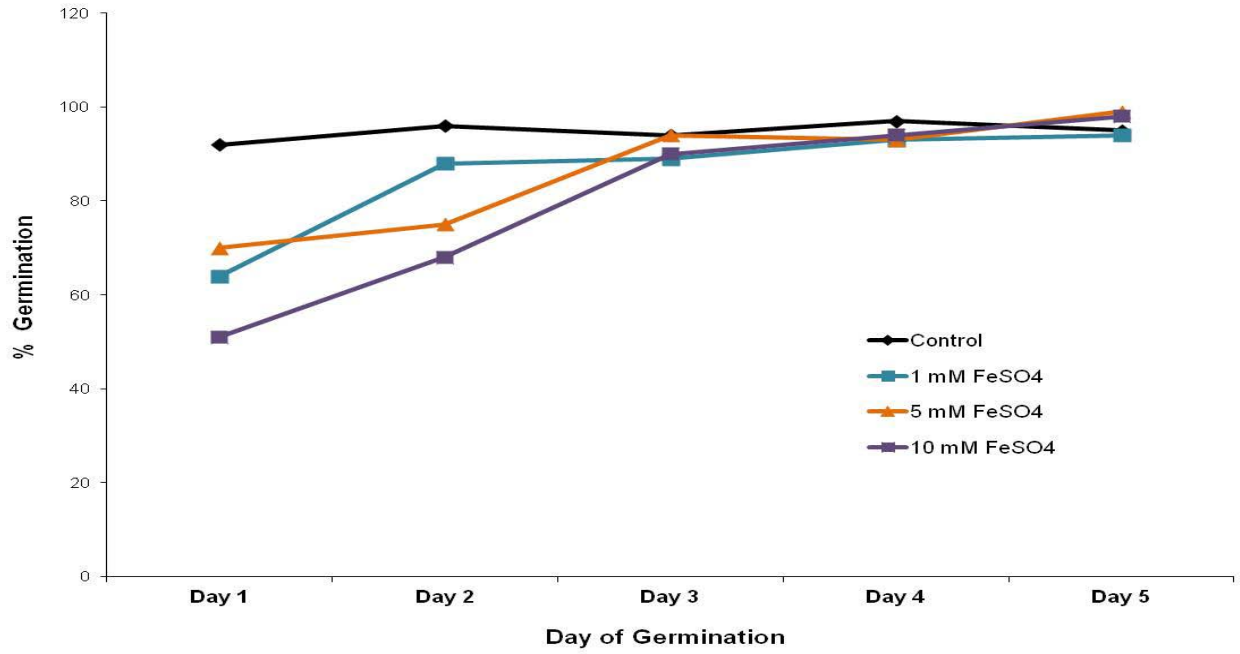


Figure 3.28: Effects of different concentrations of $FeSO_4$ on the germination levels in barley grains. The different $FeSO_4$ concentrations induced a slight decrease in the level of germination at days one and two, but by day three of germination this inhibitory effect had subsided.

When the effects on shoot emergence of 1mM, 5 mM and 10 mM $FeSO_4$ additional to 5 mM 1, 10 phenanthroline in the germination media were observed each $FeSO_4$ concentrations partially rescued shoot emergence levels in a concentration – dependent manner (Fig, 3.29). This concentration dependent recovery shows that even though iron appears to be important for shoot emergence (as it recovered some of the 1, 10 phenanthroline mediated inhibition) it is not the most important factor as did not completely recover shoot emergence to that observed in the control grains.

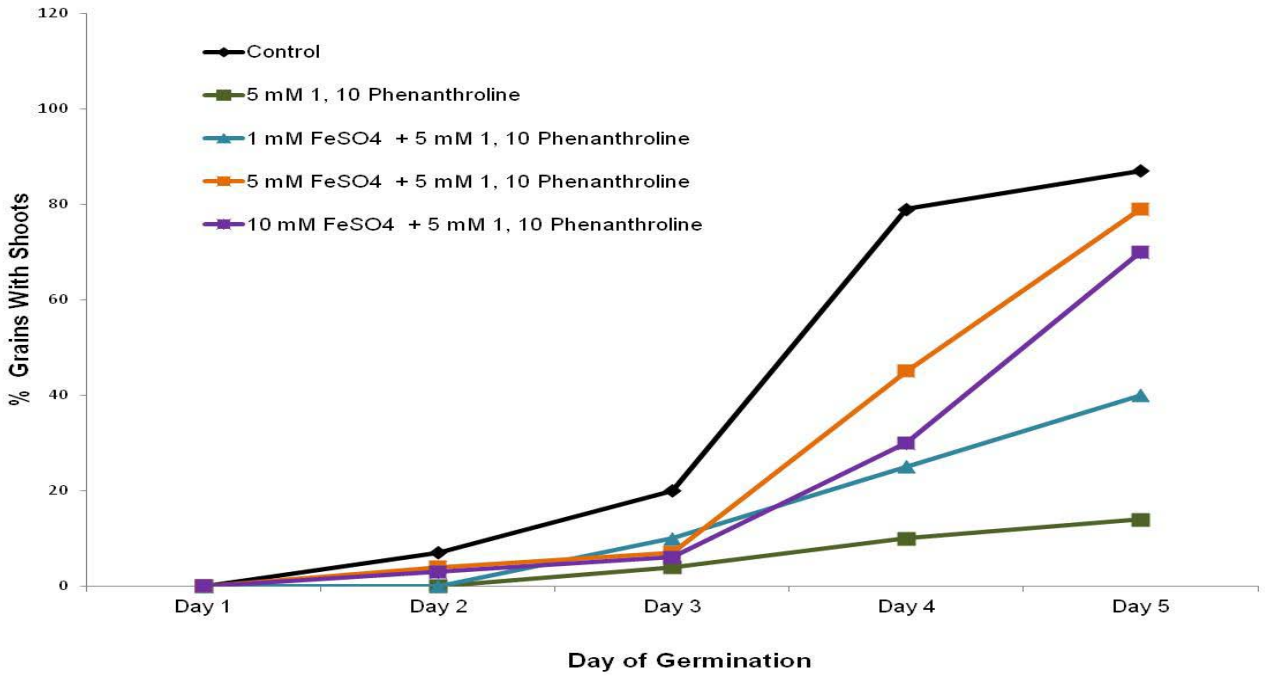


Figure 3.29: Effects of 5 mM 1, 10 phenanthroline and varying $FeSO_4$ concentrations on the shoot emergence in barley grains during germination. All three $FeSO_4$ concentrations used rescued some of the 5 mM 1, 10 phenanthroline mediated inhibition of shoot growth with 5 mM $FeSO_4$ rescuing to a greater extent than 10 mM $FeSO_4$

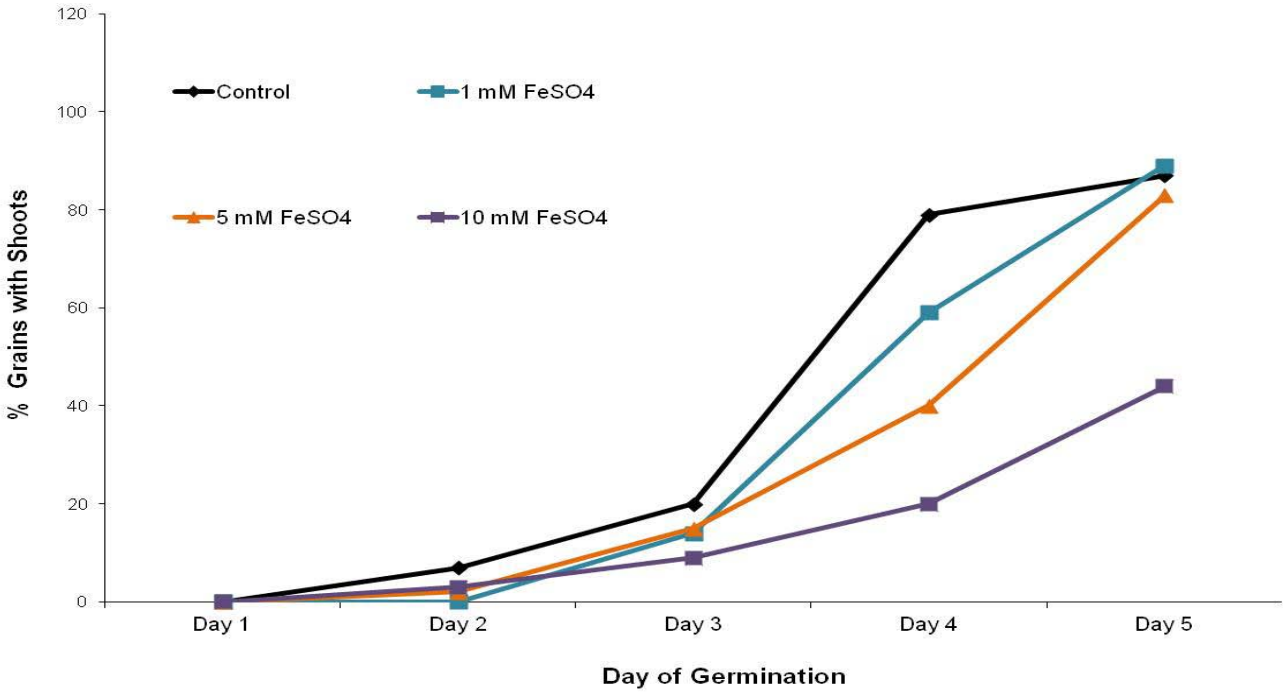


Figure 3.30: Effects of different concentrations of $FeSO_4$ on shoot emergence in germinating barley grains. All three $FeSO_4$ concentrations used had an inhibitory effect, with the degree of inhibition being $FeSO_4$ concentration dependent

When 1 mM, 5 mM and 10 mM $FeSO_4$ were added in the absence of 1, 10 phenanthroline, there was a concentration dependent decrease in shoot emergence (Fig. 3.30) with 1 mM $FeSO_4$ displaying the smallest decrease, and 10 mM $FeSO_4$ displaying the largest. These observations thus indicate iron sensitivity in the process of shoot emergence during barley grain germination.

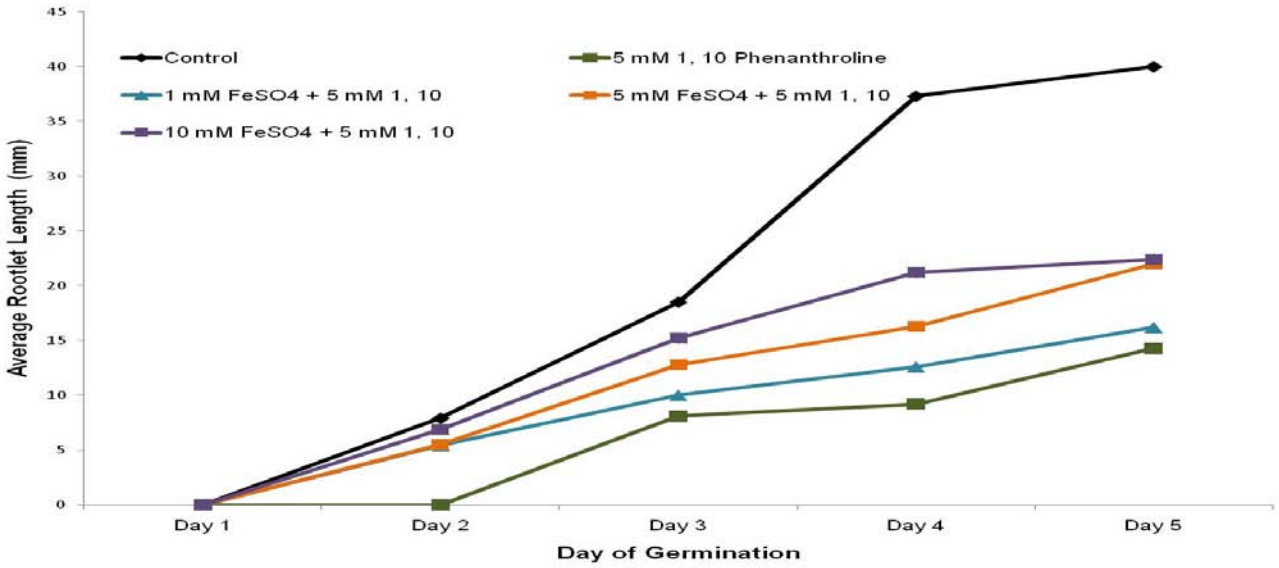


Figure 3.31: Effects of 5 mM 1, 10 phenanthroline and varying $FeSO_4$ concentrations on average rootlet length of germinating barley grains. Addition of 1 mM, 5 mM and 10 mM $FeSO_4$ along with 5 mM 1, 10 phenanthroline partially rescued average rootlet length but not to the same length as the control grains after day two of germination.

When the effects of 1, 10 phenanthroline and different $FeSO_4$ concentrations on the average rootlet length of the germinating grains were observed, it was seen that up to day two all three $FeSO_4$ concentrations showed similar rootlet lengths to the control grains (Fig. 3.31) indicating almost complete rescue of 1, 10 phenanthroline inhibition of rootlet length. However, after day two of germination the grains incubated in the different $FeSO_4$ concentrations and 1, 10 phenanthroline showed only partial recovery in rootlet length (Fig. 3.31) indicating that it is more than just the levels of Fe^{2+} within the grain that are important for rootlet growth.

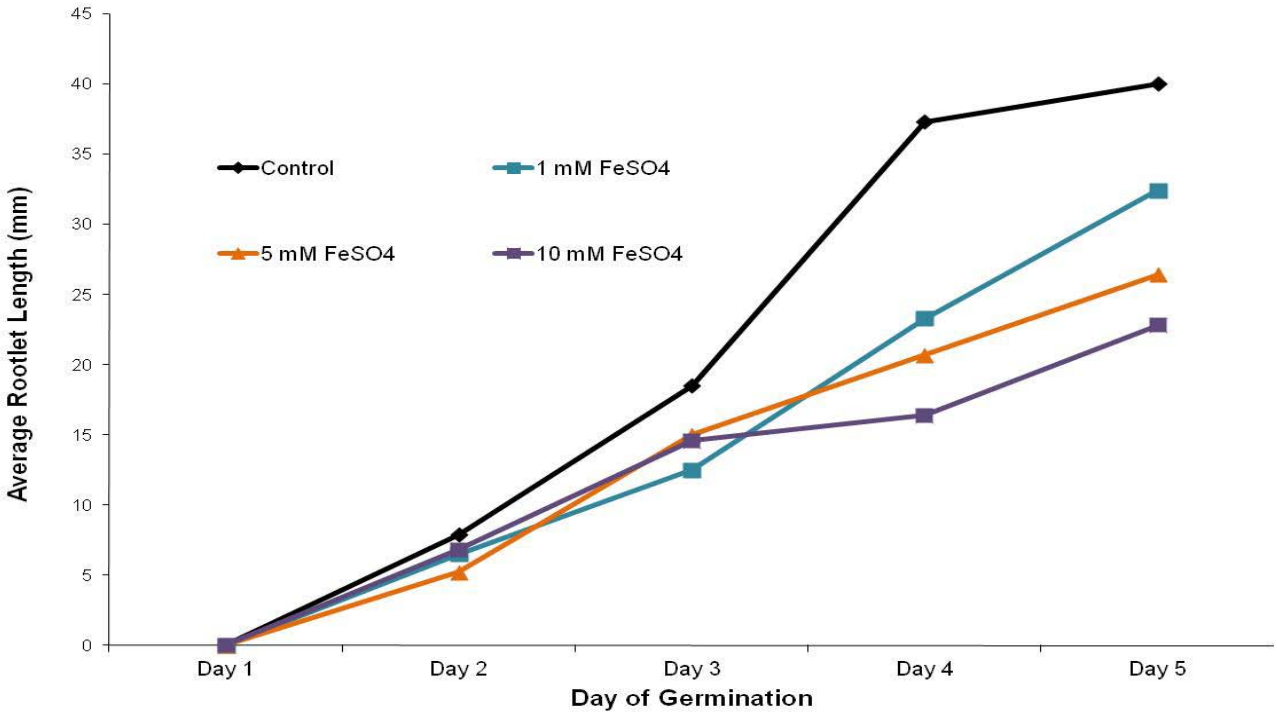


Figure 3.32: effects of different concentrations of $FeSO_4$ on average rootlet length in germinating barley grains. The addition of 1mM $FeSO_4$, 5 mM $FeSO_4$ and 10 mM $FeSO_4$ to the germination media of barley grains resulted in a reduction in average rootlet length from approximately day three of germination

When 1mM $FeSO_4$, 5 mM $FeSO_4$ and 10 mM $FeSO_4$ were added in the absence of 1, 10 phenanthroline it was observed that all three $FeSO_4$ concentrations inhibited average rootlet length from approximately day three of germination (Fig. 3.32) and that this inhibition was concentration dependent, indicating iron sensitivity in the process of rootlet growth during barley grain germination.

The effects of calcium ($CaCl_2$) on the 1, 10 phenanthroline induced inhibition of germination was investigated by germinating barley grains in the presence of both 5 mM 1, 10 phenanthroline and different $CaCl_2$ concentrations (1 mM, 5 mM and 10 mM $CaCl_2$). It was seen that (Figs. 3.33 to 3.35) when $CaCl_2$ was added along with 5 mM 1, 10 phenanthroline there was little difference in germination percentage, average rootlet length or shoot emergence levels compared to the 1, 10 phenanthroline alone grains.

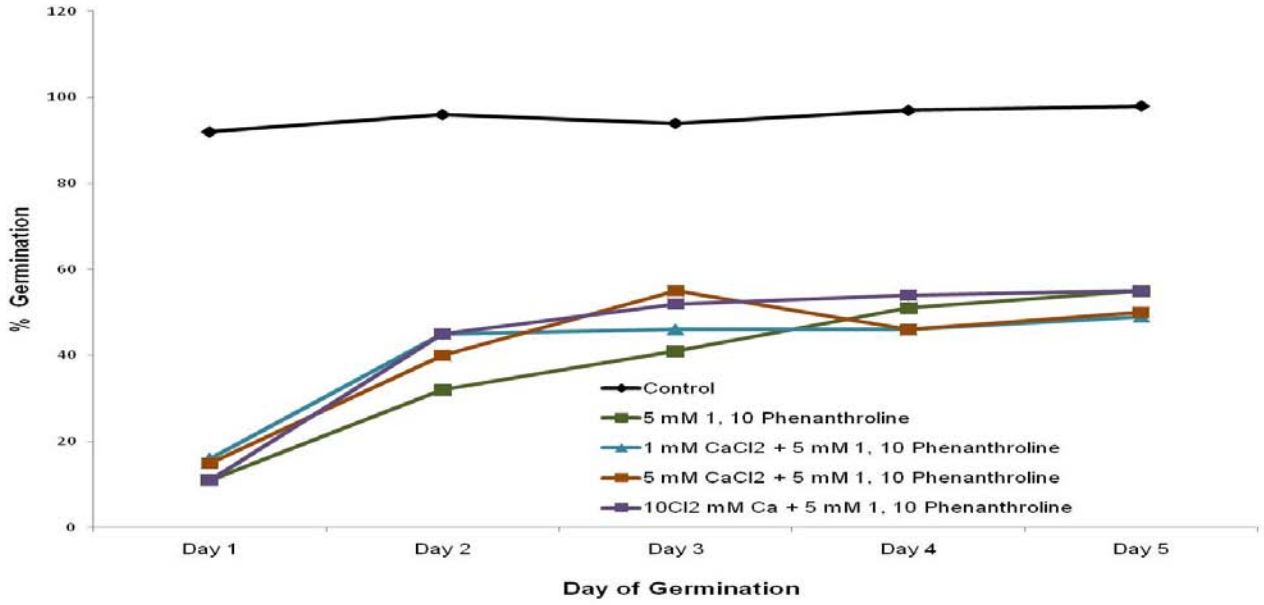


Figure 3.33: Effects of 5 mM 1, 10 phenanthroline and varying $CaCl_2$ concentrations on the germination of barley grains. The inclusion of 1 mM, 5 mM and 10 mM $CaCl_2$ into the germination media (along with 5 mM 1, 10 phenanthroline) had little effect on the 1, 10 phenanthroline induced inhibition of germination

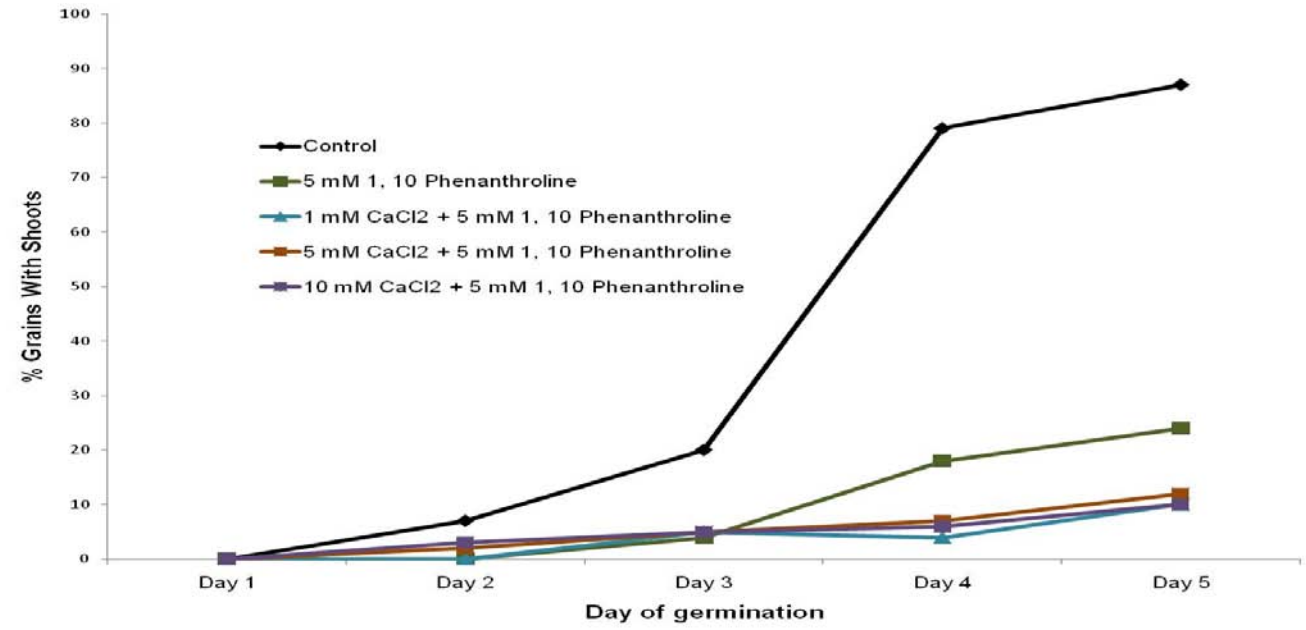


Figure 3.34: Effects of different concentrations of $CaCl_2$ on the 5 mM 1, 10 phenanthroline induced inhibition shoot emergence in germinating barley grains. The inclusion of 1 mM $CaCl_2$, 5 mM $CaCl_2$ and 10 mM $CaCl_2$ in the germination media of germination barley grains did not result in a rescue of the 1, 10 phenanthroline induced inhibition of shoot emergence

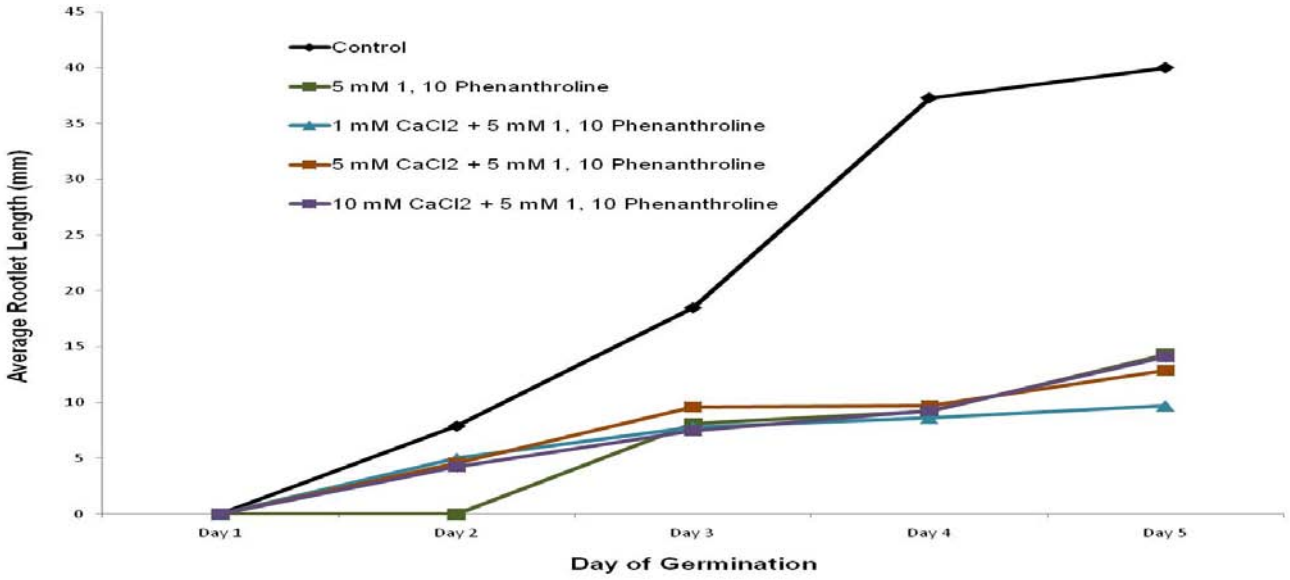


Figure 3.35: Effects of 5 mM 1, 10 phenanthroline and varying $CaCl_2$ concentrations on average rootlet length of germinating barley grains. Addition of 1 mM, 5 mM and 10 mM $CaCl_2$ with 5 mM 1, 10 phenanthroline did not rescue the inhibition of rootlet length induced by 1, 10 phenanthroline

1 mM, 5 mM and 10 mM $CaCl_2$ were then added alone, and observations made on their effects the levels of grain germination (Fig. 3.36), shoot emergence (Fig. 3.37) and the average rootlet length of the barely grains (Fig. 3.38).

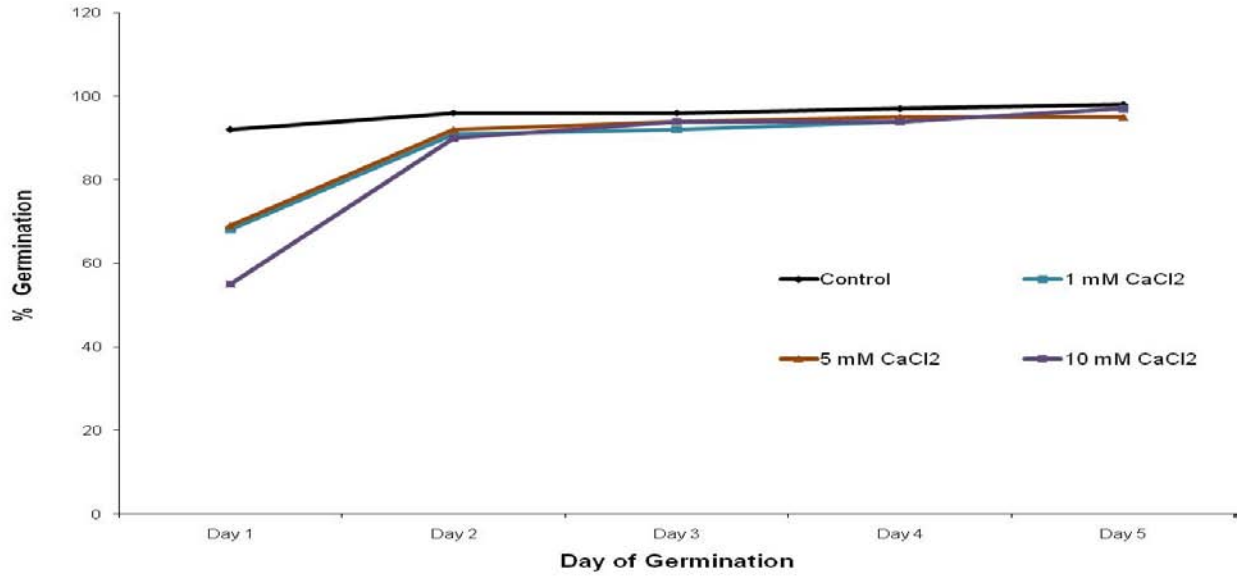


Figure 3.36: Effects of different concentrations of $CaCl_2$ on barley grain germination. The addition of 1mM $CaCl_2$, 5 mM $CaCl_2$ and 10 mM $CaCl_2$ to the germination media of barley grains resulted in little difference in the overall levels of germination except for day one where all $CaCl_2$ concentrations resulted in a small decrease in the levels of germination

The addition of 1 mM, 5 mM and 10 mM $CaCl_2$ alone to the germination media of barley grains decreased germination during day one (Fig. 3.36) but by day two germination levels were approximately the same as the control grains in all three $CaCl_2$ concentrations, indicating that the overall process of germination only be sensitive to external calcium levels during early germination.

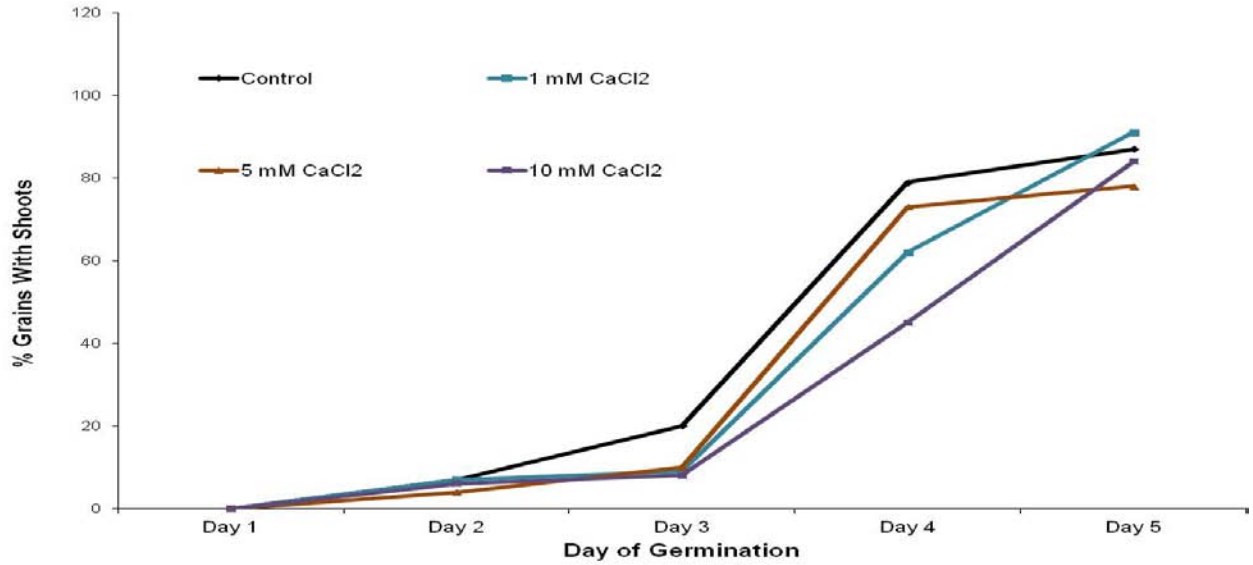


Figure 3.37: Effects of different concentrations of $CaCl_2$ on shoot emergence in barley grains during germination. When the different $CaCl_2$ concentrations were added alone there was a small decrease in the shoot emergence levels with all three $CaCl_2$ concentrations from about day two of germination

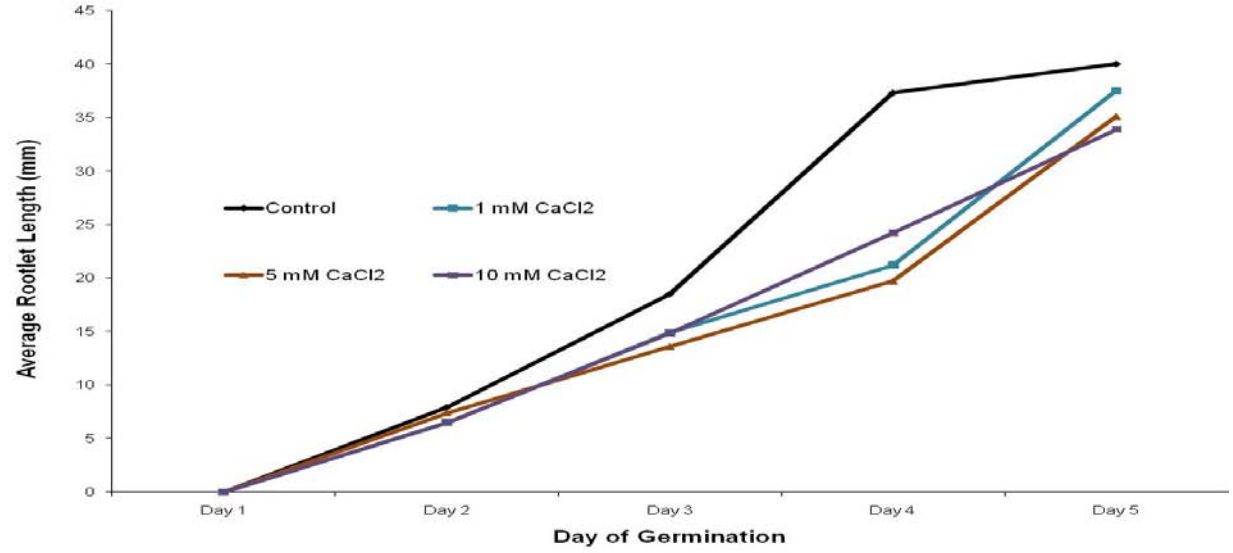


Figure 3.38: Effects of different concentrations of $CaCl_2$ on the average rootlet length of barley grains during germination. From approximately day two of germination all three $CaCl_2$ concentrations slightly reduced the barley grain average rootlet length

When average rootlet length and shoot emergence observation for the different $CaCl_2$ concentrations were compared to the controls, a reduction in both parameters was observed when calcium was included in the medium (Figs. 3.37 and 3.38). Both the decrease in average rootlet length and the decrease in the levels of shoot emergence were seen from approximately day two of germination (Figs. 3.37 and Fig. 3.38) and continued throughout the length of the study demonstrating that these two processes may be calcium sensitive.

The results shown in Figs. 3.25 to 3.38 could show that it is not just divalent cations that reverse the 1, 10 phenanthroline mediated inhibition of germination, but that only certain divalent cations will do so; these are possibly transition metal ions as both iron and zinc are transition metals whereas calcium is not. To further investigate the reversal of 1, 10 phenanthroline mediated germination inhibition by the transition metals and non transition metals, two further members of these elemental groups were investigated (Figs. 3.39 to 3.42); magnesium (a non – transition metal divalent cation) and manganese (a transition metal divalent cation). Also, to investigate the significance of the counter ion (for example chloride or sulphate) on the reversal of 1, 10 phenanthroline mediated inhibition of germination, two different magnesium compounds were investigated – $MgCl_2$ and $MgSO_4$. The effects of these cations, when added to the germination media at 5 mM without 1, 10 phenanthroline were also investigated (Figs. 3.44 to 3.46).

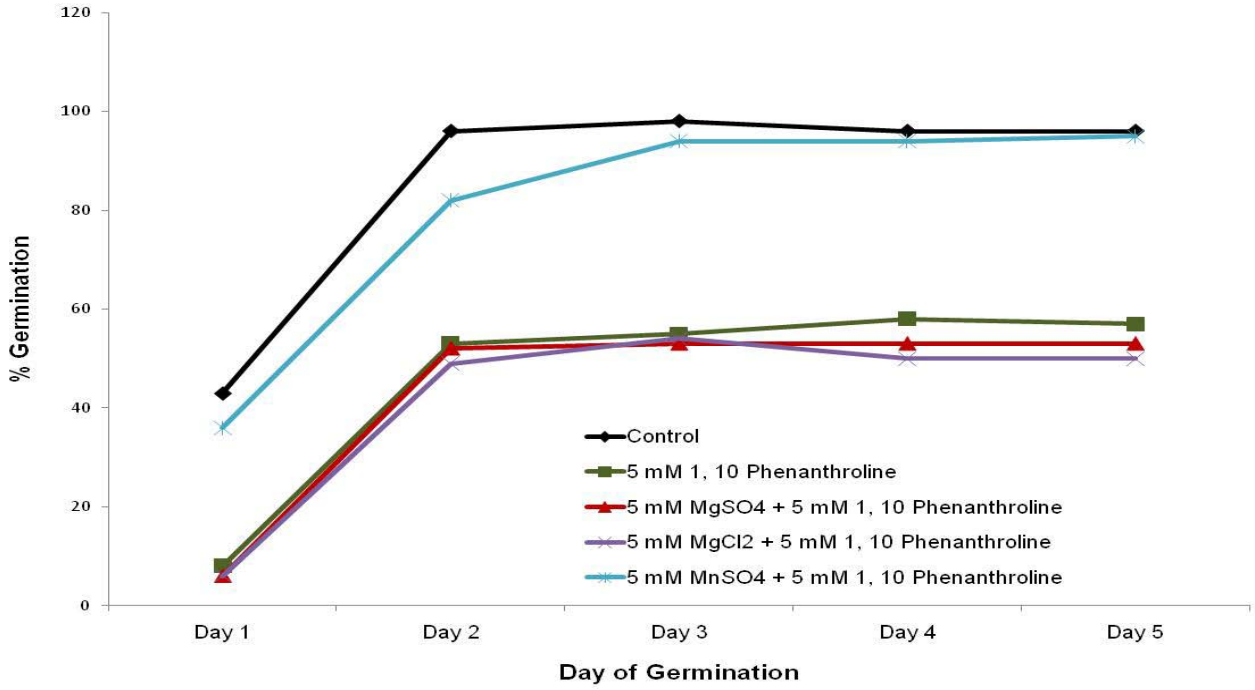


Figure 3.39: Effects of $MgCl_2$, $MgSO_4$ and $MnSO_4$ on the 1, 10 phenanthroline mediated inhibition of germination in barley grains. The addition of 5 mM $MgCl_2$ and $MgSO_4$ had little effect on the levels of germination in the presence of 5 mM 1, 10 phenanthroline. But the addition of 5 mM $MnSO_4$ with 5 mM 1, 10 phenanthroline rescued germination almost completely

The only divalent cation of the two to rescue the levels of germination in the presence of 5 mM 1, 10 phenanthroline (Fig. 3.39) was manganese ($MnSO_4$). This was also seen for shoot emergence (Fig. 3.40) where the addition of 5 mM $MnSO_4$ along with 5 mM 1, 10 phenanthroline to the germination media rescued the shoot emergence to about 75 % of the control value by day five of germination. However, it was not until day four of germination that manganese produced any recovery in the average rootlet length measurements (Fig. 3.41) further demonstrating that different processes within germination have differing requirements for divalent cations.

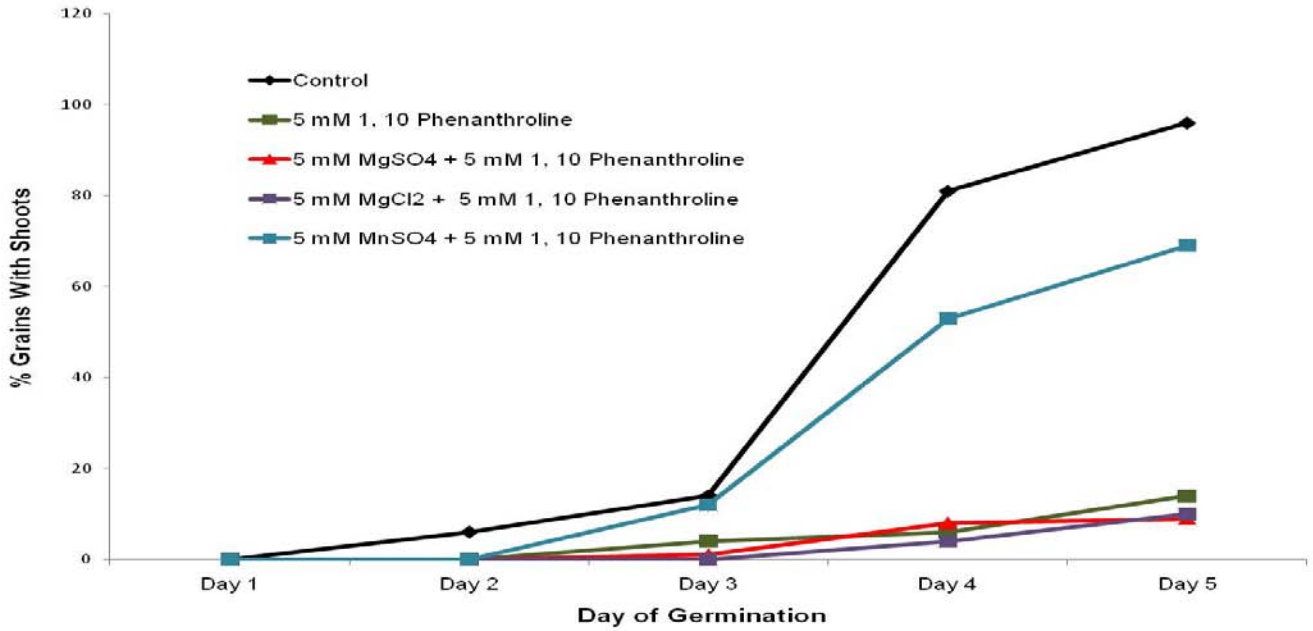


Figure 3.40: Effects of $MgCl_2$, $MgSO_4$ and $MnSO_4$ on the5 mM 1, 10 phenanthroline mediated inhibition of shoot emergence in germinating barley grains. Adding 5 mM $MgCl_2$ or 5 mM $MgSO_4$ along with 5 mM 1, 10 phenanthroline to the germination media of barley grains made little difference to the 1, 10 phenanthroline mediated inhibition of shoot emergence. However, adding 5 mM $MnSO_4$ along with 5 mM 1, 10 phenanthroline reversed this inhibition by approximately 75 % by day five of germination

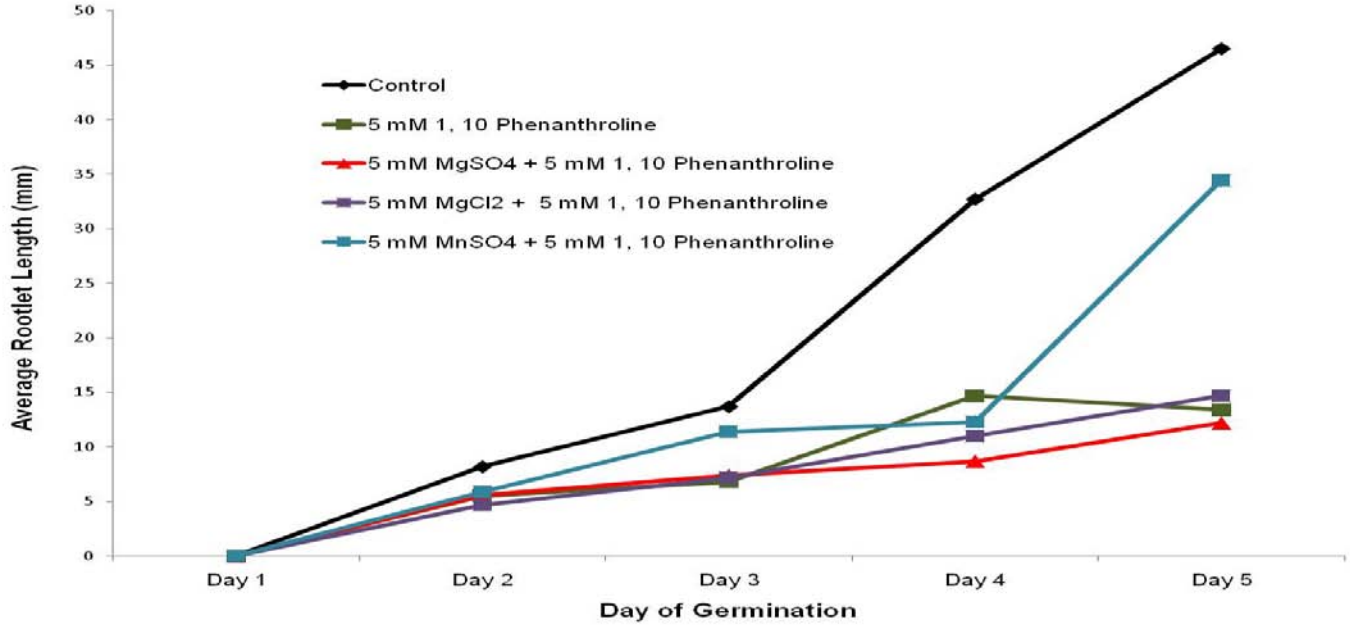


Figure 3.41: Effects of $MgCl_2$, $MgSO_4$ and $MnSO_4$ on the 1, 10 phenanthroline mediated inhibition of average rootlet length in germinating barley grains. None of the divalent cations added reversed the 1, 10 phenanthroline mediated inhibition of rootlet length apart from $MnSO_4$ at day five of germination

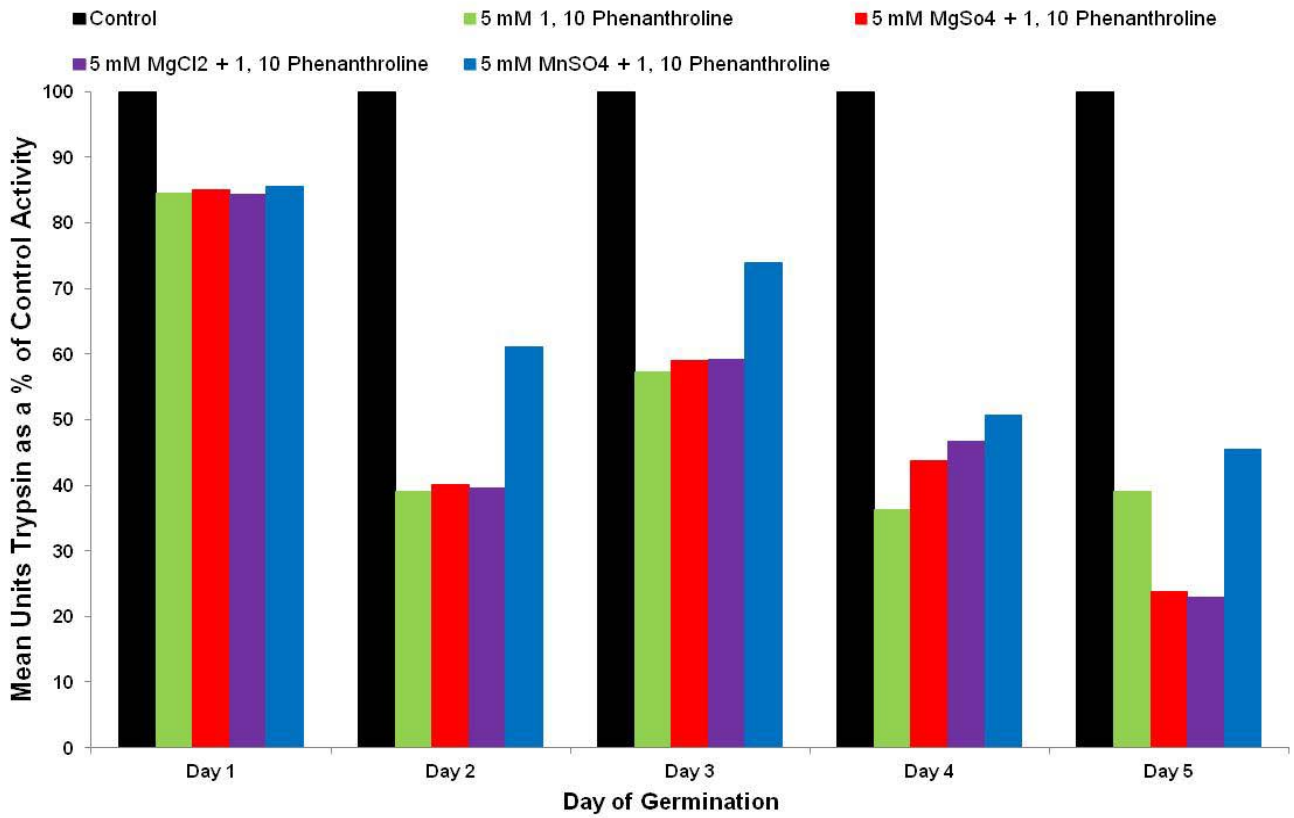


Figure 3.42: Effects of 1, 10 phenanthroline with $MgSO_4$, $MgCl_2$ and $MnSO_4$ on the protease activity present in barley grains during germination as a % of control values. None of the different divalent cations added fully rescued the 5 mM 1, 10 phenanthroline mediated inhibition of protease activity during barley grain germination

The effects of 5 mM $MgSO_4$, 5 mM $MgCl_2$ and 5 mM $MnSO_4$ on the levels of protease activity during germination in the presence of 5 mM 1, 10 phenanthroline was also investigated (Fig. 3.42). This investigation showed that neither divalent cations tested fully rescued the 1, 10 phenanthroline mediated inhibition of protease activity to control values within the germinating barley grains, but that the addition of 5 mM $MnSO_4$ was the most effective, rescuing protease activity levels to the greatest extent, and rescuing from days two to five (Fig. 3.42). The inclusion of $MgSO_4$ and $MgCl_2$ along with 1, 10 phenanthroline did produce a small increase in protease activity levels but only at day four of germination (Fig. 3.42). Thus some of the metalloproteases active during barley grain germination may be manganese dependent, but it is unlikely that any of them are magnesium dependent.

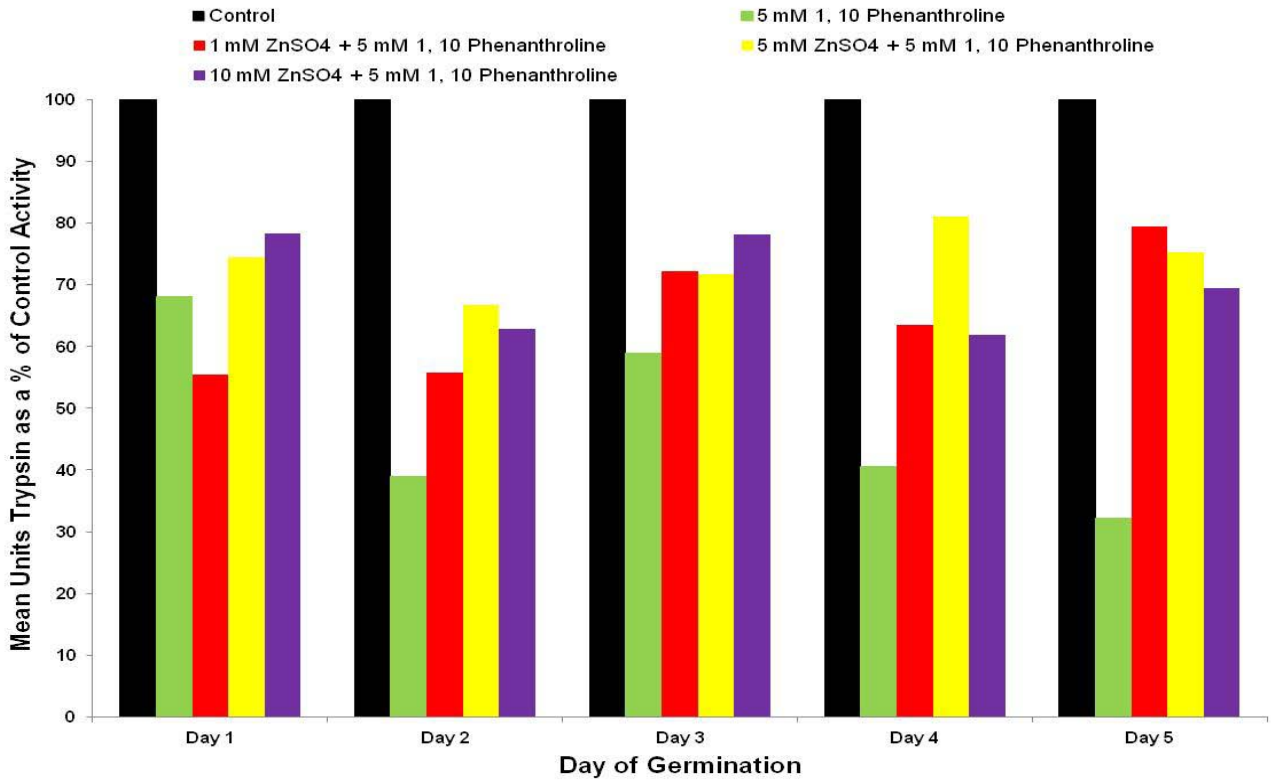


Figure 3.43: Effects of 1, 10 phenanthroline with different concentrations of $ZnSO_4$ on the protease activity present in barley grains during germination as a % of control values. None of the $ZnSO_4$ concentrations investigated fully rescued the 5 mM 1, 10 phenanthroline mediated inhibition of protease activity during germination. However, the different $ZnSO_4$ concentrations rescued a greater percentage of protease activity during the later stages of germination

Since the majority of metalloproteases are known to be zinc dependent (Beynon & Boyd, 2001), the effects of the addition of different concentrations of $ZnSO_4$ along with 5 mM 1, 10 phenanthroline on the levels of protease activity in germinating barley grains was investigated (Fig. 3.43). This study showed that the addition of all three $ZnSO_4$ concentrations rescued protease activity levels from day one of germination (Fig. 3.43), and that the degree of rescue increased as germination progressed. However, on day one of germination the addition of 1 mM $ZnSO_4$ along with 5 mM 1, 10 phenanthroline was seen to inhibit protease activity levels to a greater extent than with 1, 10 phenanthroline alone (Fig. 3.43). Since this was only observed at day one and that none of the other $ZnSO_4$ concentrations were seen to inhibit protease activity levels, this result is probably due to problems with experimental technique such as pipetting errors rather than any inhibitory effect of zinc addition on protease activity.

Since $MnSO_4$ reversed the 1, 10 phenanthroline mediated inhibition of germination (and magnesium did not), $MnSO_4$ was added alone (in varying concentrations) to the germination media of barley grains to investigate its effects on germination in the absence of 1, 10 phenanthroline (Figs. 3.44 to 3.46).

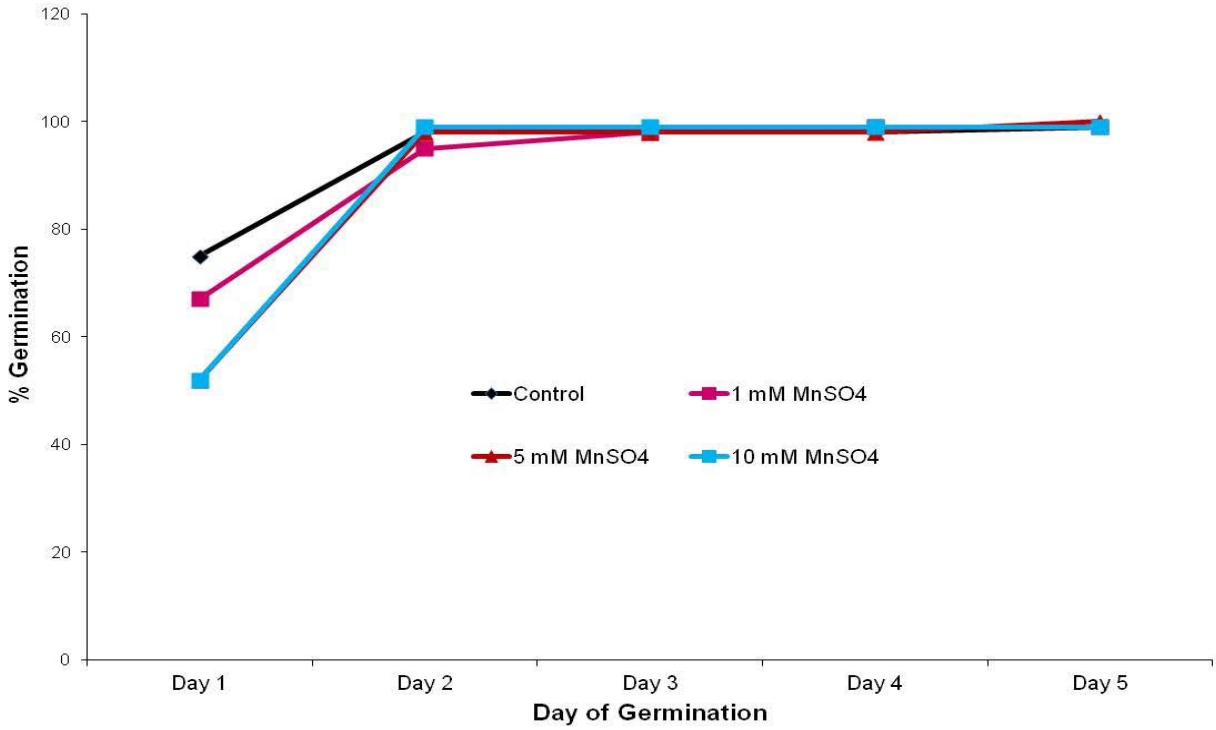


Figure 3.44: Effects of different concentrations of $MnSO_4$ on barley grain germination. At day one of germination all three $MnSO_4$ concentrations slightly reduced germination. This inhibition of germination was recovered by day two.

When 1 mM $MnSO_4$, 5 mM $MnSO_4$ and 10 mM $MnSO_4$ were added in the absence of 5 mM 1, 10 phenanthroline to the germination media of ungerminated barley grains, none of the three $MnSO_4$ concentrations affected grain germination (Fig. 3.44) after day one of germination. In the shoot emergence observations the three $MnSO_4$ concentrations had little effect until approximately day four of germination (Fig. 3.45) where the grains germinated in the presence of 1 mM, 5 mM and 10 mM $MnSO_4$ showed only a very slight reduction in the numbers of grains with visible shoots.

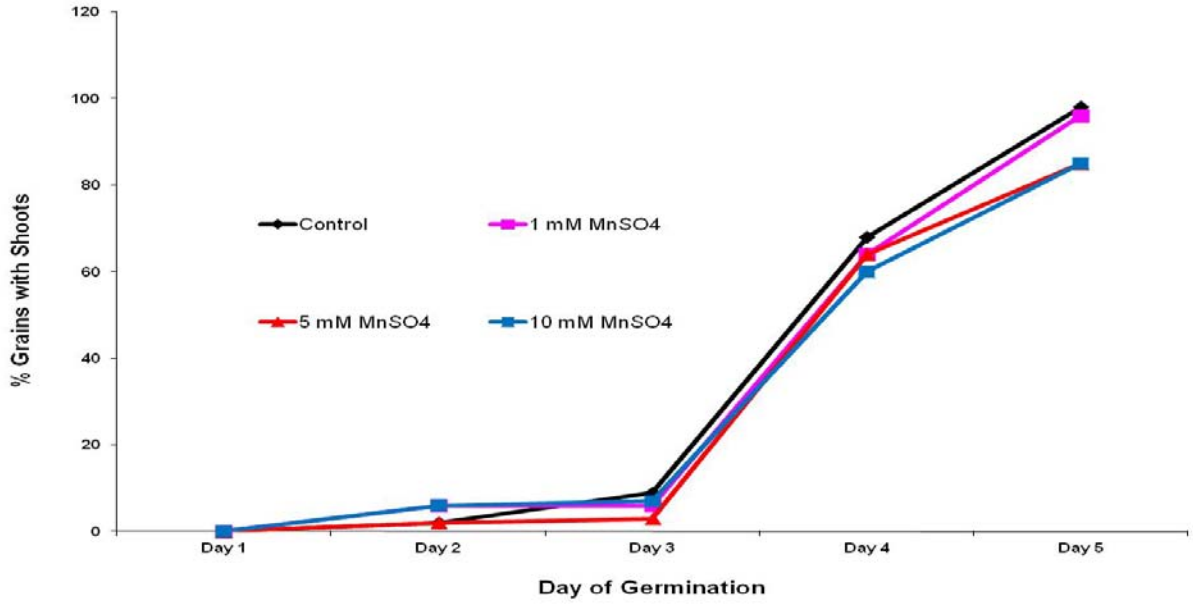


Figure 3.45: Effects of different concentrations of $MnSO_4$ on shoot emergence in germinating barley grains. None of the three $MnSO_4$ concentrations had an effect on shoot emergence during germination until approximately day four of germination relative to control grains

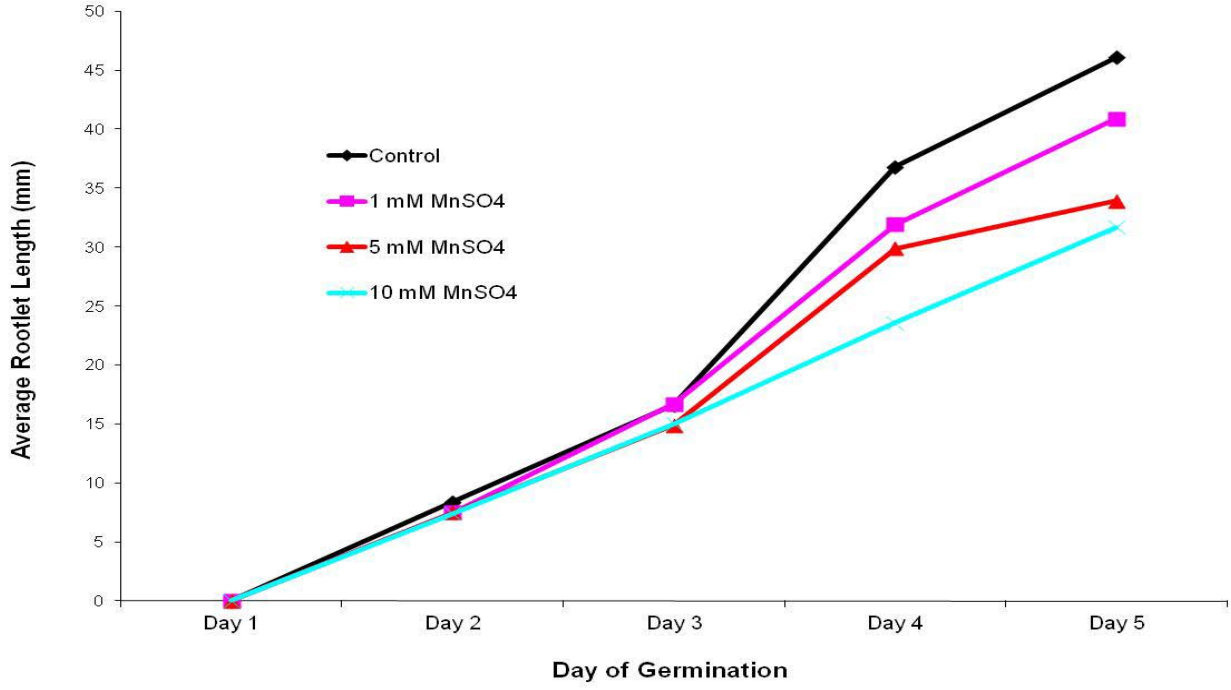


Figure 3.46: Effects of different concentrations of $MnSO_4$ on the average rootlet length of germinating barley grains. From approximately day three of germination all three $MnSO_4$ concentrations reduced the average rootlet length of the barley grains in a concentration dependent manner

When average rootlet length measurements were taken a small inhibition of rootlet length could be observed from approximately day three of germination in a $MnSO_4$ concentration dependent manner (Fig. 3.46), indicating that rootlet growth is sensitive to external manganese levels.

Overall, the divalent cation and 1, 10 phenanthroline studies could show that transition metal ions such as zinc, iron and manganese are important components of germination and that there are temporal and germination process dependent variations in the requirements and sensitivities of these transition metal ions.

3.5: Proteases & Starch Degrading Enzymes

3.5.1: Limit Dextrinase

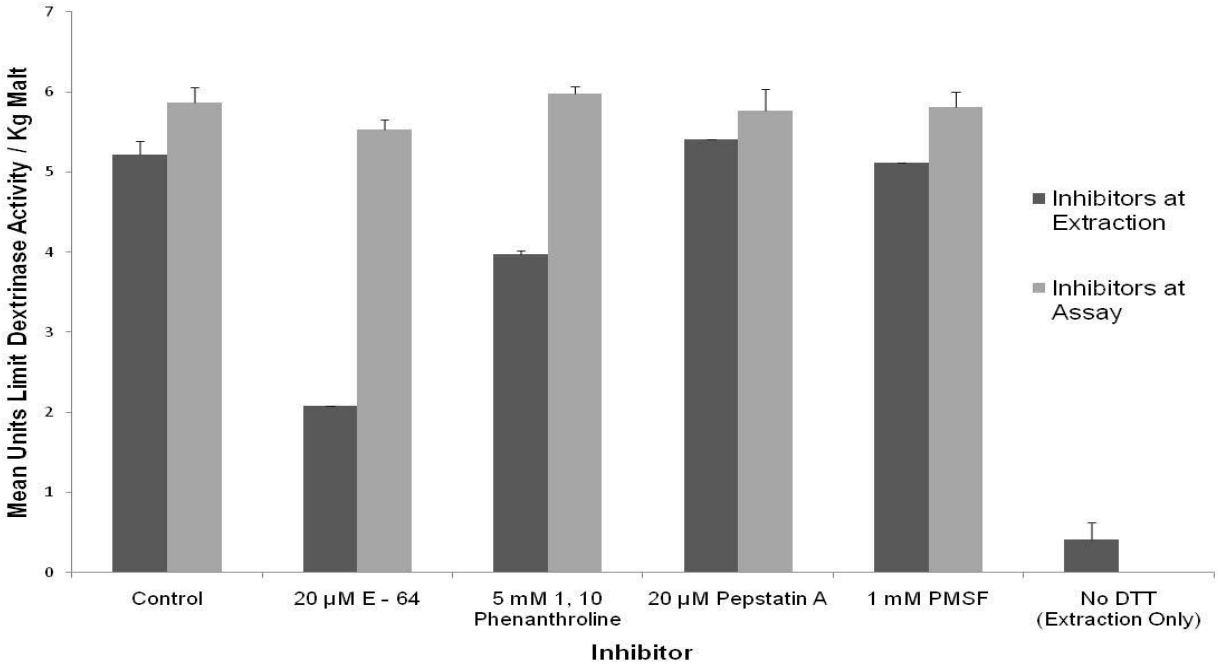


Figure 3.47: Effects of class specific protease inhibitors on the activity of limit dextrinase. At the extraction stage the greatest effect of inhibitor inclusion was observed at the assay stage with E – 64, followed by 1, 10 phenanthroline. DTT is required during limit dextrinase extraction only so it was not investigated during the assay stage. Error bars indicate standard deviation, n = 3

Limit dextrinase is one of the major starch degrading enzymes active in barley grains during germination. During grain development limit dextrinase exists in an inactive "bound" form complexed with a proteinaceous inhibitor molecule (MacGregor, 2004). During germination this inhibitory complex is broken down by reducing conditions (Cho et al, 1999) and / or cysteine protease mediated degradation of the proteinaceous inhibitor (Longstaff & Bryce, 1993) liberating active limit dextrinase.

To investigate the role of proteases in the activation of limit dextrinase *in vitro*, limit dextrinase activity assays were carried out on four day kilned malt in the presence of class specific protease inhibitors and the reducing agent DTT, added during either the extraction or assay stage (no DTT was added during the assay stage) of the assay. As the results in Fig. 3.47 clearly show, no significant inhibition of limit dextrinase activity was observed by inclusion of the inhibitors during the activity assay, indicating that the enzyme undergoes no proteolytic regulation after extraction and that the inhibitors do not interfere with the assay. However, when the inhibitors and DTT were added at the extraction stage it becomes evident that the activation of limit dextrinase is performed largely by reducing conditions and cysteine proteases (indicated by the large amount of inhibition achieved by the inclusion of E – 64 in the extraction media, and also by the absence of DTT from the extraction media), and to a smaller extent by metalloproteases as shown by the reduction in activity observed by the addition of 1, 10 phenanthroline to the extraction media. Since these results fit well with the literature (Longstaff & Bryce, 1993; MacGregor, 2004) no further studies involving limit dextrinase were carried out.

3.5.2: α - Amylase

α – amylase is one of the few barley grain enzymes to be active during germination which can initiate native starch hydrolysis (Georg – Kraemer et al, 2001).

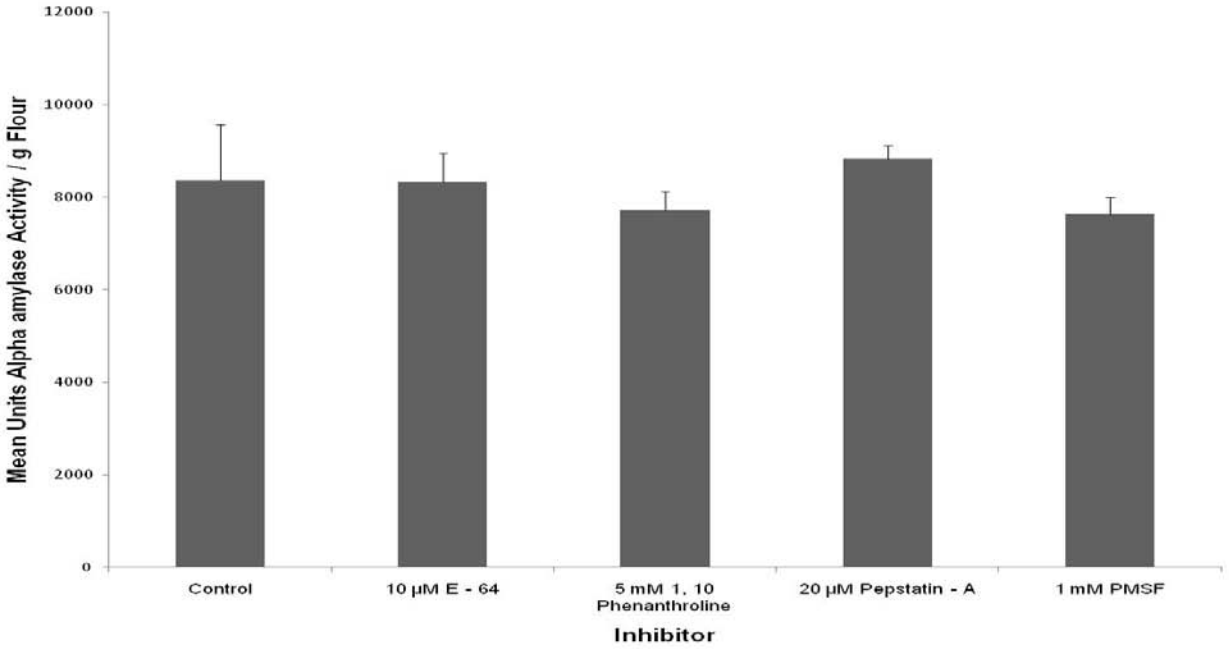


Figure 3.48: Effects of class specific protease inhibitors on the activity of α – amylase in four day kilned malt when added at the assay stage. Class specific protease inhibitor inclusion in the assays of α – amylase had little effect on the overall activity of α – amylase. Error bars indicate standard deviation, n = 3

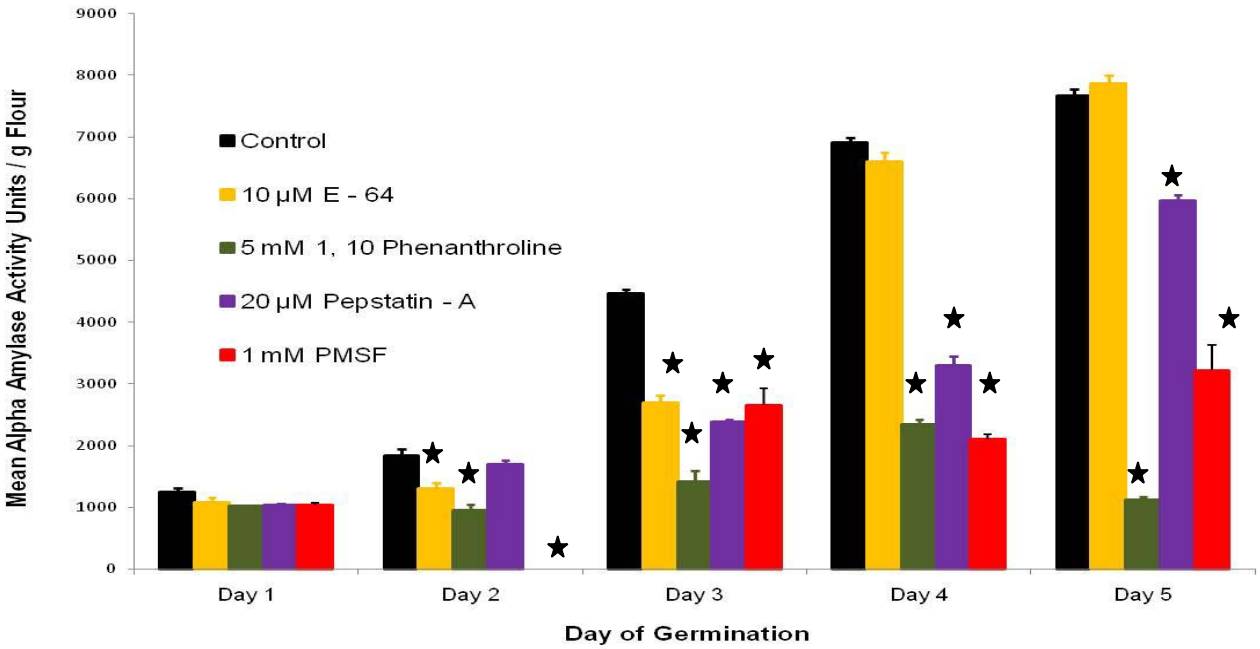


Figure 3.49: Effects of class specific protease inhibitors on the activity of α – amylase during germination. At day one of germination none of the inhibitors produced a significant difference in the levels of α – amylase activity. Grains germinated in the presence of 1 mM PMSF and also 5 mM 1, 10 phenanthroline showed significant ($p = < 0.05$) decreases in α - amylase activity from day two all the way through to day five of germination. Error bars represent standard deviation, n = 3, ★ = Significant differences ($p = < 0.05$) in α - amylase activity between the control and inhibitor on that particular day of germination

When class specific protease inhibitors were added to α – amylase assays at the assay stage one – way ANOVA and Bonferroni post – hoc analysis showed that there were no significant differences ($p = < 0.05$) between the control α – amylase activity levels and those with the different inhibitors present (Fig. 3.48). However, when grains were germinated in the presence of the different class specific protease inhibitors, the levels of α – amylase activity fell in the presence of all inhibitors on certain germination days (Fig. 3.49). The most notable decrease was that observed on day two in the presence of 1 mM PMSF where 100 % of α – amylase activity was inhibited. This total inhibition was relieved by day three but the levels of α – amylase activity were still significantly lower than the control values throughout the investigation (Fig. 3.49) indicating that serine proteases could have a role in positively regulating α – amylase activity in barley grains during germination. This potential positive regulation of α – amylase activity was also observed in the presence of the class specific aspartate protease inhibitor pepstatin A (Fig. 3.49). In the presence of 20 μM pepstatin A the levels of α - amylase activity measured was significantly reduced on days three to five of germination pointing to a potentially similar role in the regulation of α – amylase activity to that of the serine class proteases. The presence of 10 μM E – 64 in the barley grain germination media produced a significant decrease in α – amylase activity at days two and three of germination only (Fig. 3.49) demonstrating a role for the cysteine protease, also, in the positive regulation of α – amylase activity.

The large and significant reduction in the activity of α – amylase (from days two to five of germination) in the presence of the metalloprotease inhibitor 1, 10 phenanthroline is probably most likely to be a result of the inhibition of germination caused by the inclusion of 1, 10 phenanthroline in the germination media (as α – amylase is synthesised during grain germination) and also possibly by the chelation of divalent cations by 1, 10 phenanthroline as α – amylase requires Ca^{2+} ions for its activity.

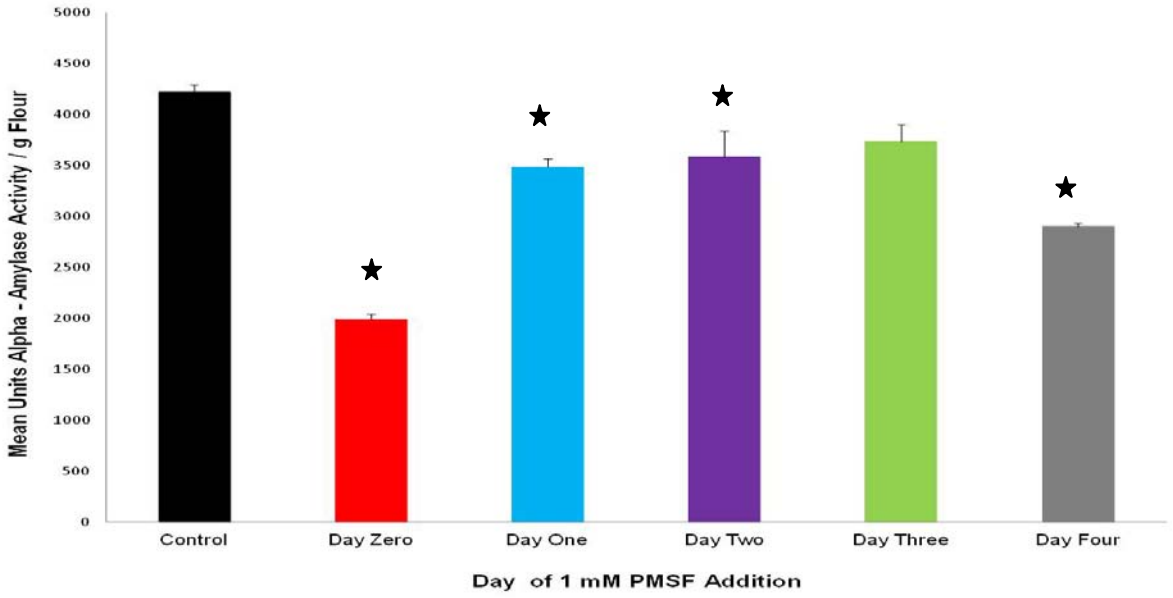


Fig 3.50: Effect of adding 1 mM PMSF at different days of germination on the activity of α – amylase. The addition of 1 mM PMSF to the germination media of barley grains at different days of germination produced a significant (p = < 0.05) reduction in the activity of α – amylase at each day of addition except for day three, where no significant differences were observed (p = < 0.05). ★ = p value of less than 0.05. Error bars represent standard deviation, n = 3.

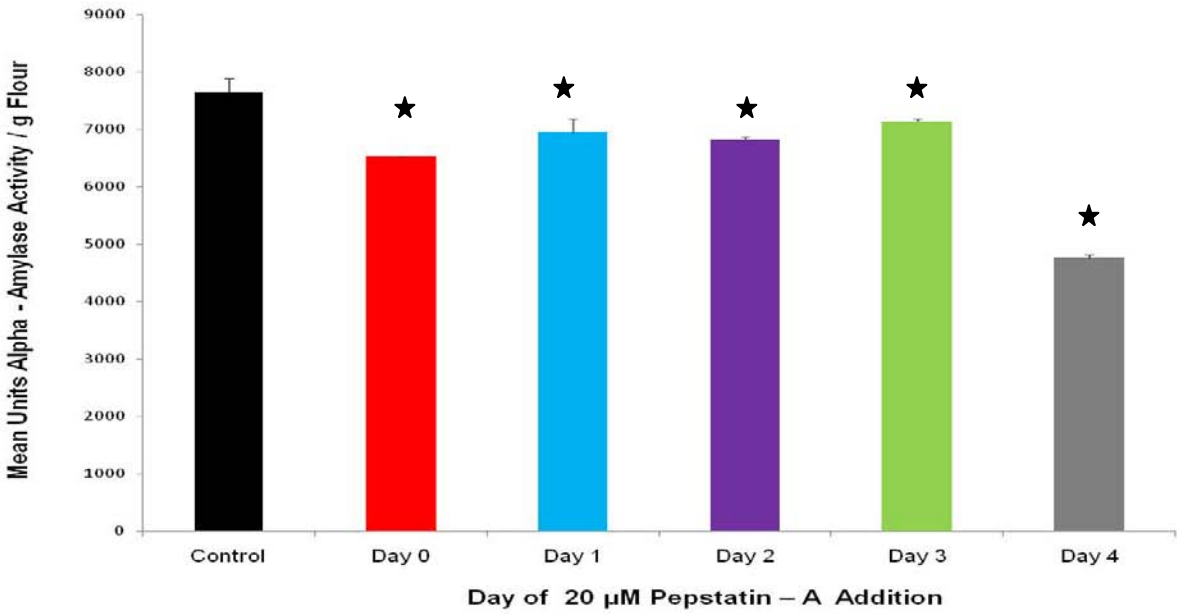


Fig 3.51: effect of adding 20 µM Pepstatin A at different days of germination on the activity of α – amylase. The addition of 20 µM pepstatin - A to the germination media of barley grains at different days of germination produced a significant (p = < 0.05) reduction in α – amylase activity at each day of addition. ★= p value of less than 0.05. Error bars represent standard deviation, n = 3.

To further investigate the effects of PMSF and pepstatin A on the activity of α – amylase, investigations were carried out where 1 mM PMSF and 20 μM pepstatin A were added, to separate grain batches, at different days of germination, then assayed at day five of germination for α – amylase activity (Figs. 3.50 and 3.51). These investigations showed that the addition of the inhibitors produced the largest decrease in α – amylase activity with 1 mM PMSF at day zero (i.e. when PMSF was added at the very beginning of the experiment) and at day four for pepstatin A, with the addition of PMSF at day zero having the greatest inhibitory effect, inhibiting approximately 45 % of α – amylase activity as opposed to only approximately 27 % for day four 20 μM pepstatin A addition. This could show that the presence of the serine class proteases could be important for the activity of α - amylase from the beginning of germination, whereas the aspartate class proteases could be more important during the later stages of germination. However, it should be noted that to avoid any potential dehydration stress, more water (and inhibitor) was added at day three of germination to the day zero and day one grains. This extra dose of inhibitor could have resulted in an exaggeration of the effects of the inhibitors in the day zero and day one grains compared to those not receiving the extra water. However, it should also be noted that PMSF has a short half – life in aqueous solution (just under two hours at pH 7 at 25 ^{o}C (James, 1978)) thus it is probable that the mechanism of α – amylase inhibition induced by the addition of PMSF to the germination media occurs over a short time period therefore, the inhibition observed by the addition of PMSF from day zero would already have occurred by day three when the extra dose was added.

With the potential of the serine and aspartate class proteases being positive regulators of α – amylase activity in mind, western blot analysis was carried out in order to investigate whether the two protease classes had a role in the degradation of α – amylase inhibitors or in the regulation of the actual amounts of α – amylase protein present in the grains (Fig. 3.52). If the two protease classes were involved in the degradation of α – amylase inhibitors then the western blot would be expected to show little difference in the levels of α – amylase protein present between the control and inhibitor germinated grains. However, as Fig. 3.52 shows, grains germinated in the presence of 1 mM PMSF (serine proteases inhibited), 20 μM pepstatin A (aspartate proteases inhibited) and both 1mM PMSF and 20 μM pepstatin A together displayed reduced amounts of α – amylase protein, showing that both serine and

aspartate class proteases are positive regulators of the amounts of α – amylase present in barley grains during germination.

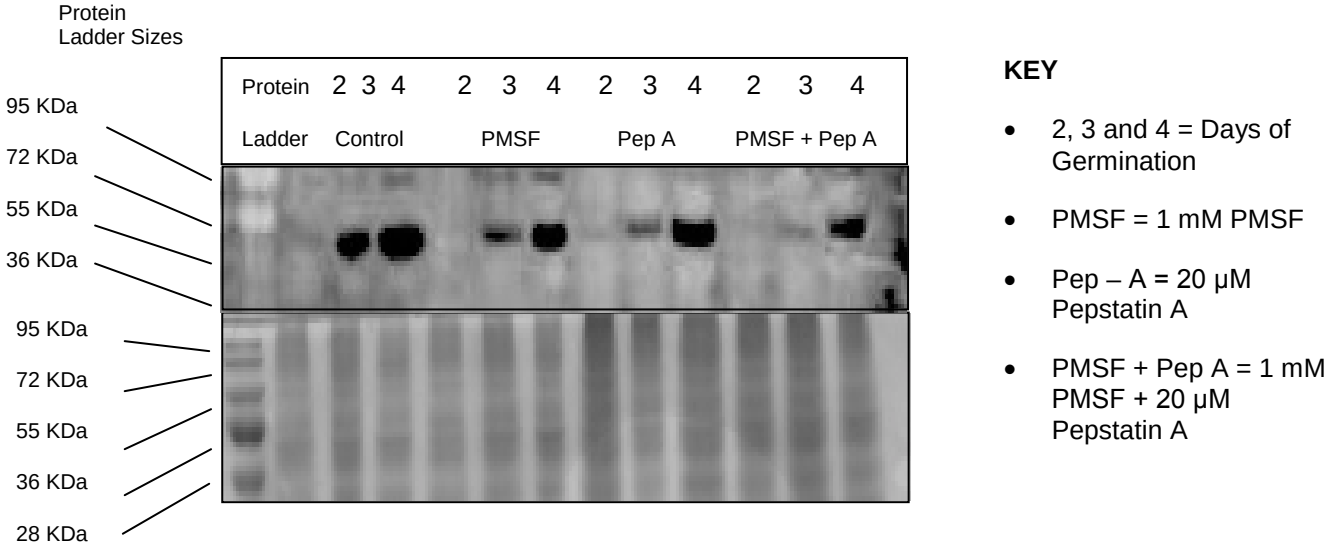


Figure 3.52: Western blot analysis of the effects of 1 mM PMSF, 20 µM pepstatin A, and 1 mM PMSF + 20 µM pepstatin A on the amount of α – amylase present in germinating barley grains. Top panel, signal from hybridised anti – α – amylase antiserum, lower panel, duplicate Coomassie blue stained SDS – PAGE to show protein loading levels. In the presence of 1 mM PMSF, 20 µM pepstatin A and both inhibitors together there was a reduction in the amount of α – amylase present in the grain during germination. This reduction is most pronounced in the presence of both 1 mM PMSF and 20 µM Pepstatin A

To investigate whether this positive regulation of the amount of α – amylase protein present in the grains was due to the involvement of the proteases in the gibberellic acid induction of α – amylase synthesis further germination studies were carried out. In these studies barley grains were germinated in the presence of the two inhibitors (added to separate grain sets) and also GA_3 (Figs. 3.53). As the data in Fig. 3.53 shows, the inhibition of α – amylase activity in the presence of both inhibitors was rescued by the inclusion of 25 µM GA_3. Furthermore, adding GA_3 together with 1 mM PMSF, 20 µM pepstatin A and PMSF and pepstatin A enhanced α – amylase activity to levels similar to, or significantly higher than the control values for each day of germination, but not as high as the levels seen when GA_3 was added on its own (Fig. 3.53). This indicates that the serine and aspartate class proteases are not primarily involved in the perception of gibberellic acid by the aleurone layer or any events downstream of this because if they were, the inclusion of GA_3 would not have rescued the levels of α – amylase activity. They could however be involved in the upstream

events of GA_3 signalling, such as gibberellic acid synthesis, secretion by the embryo, or transportation through the scutellum and into the aleurone layer.

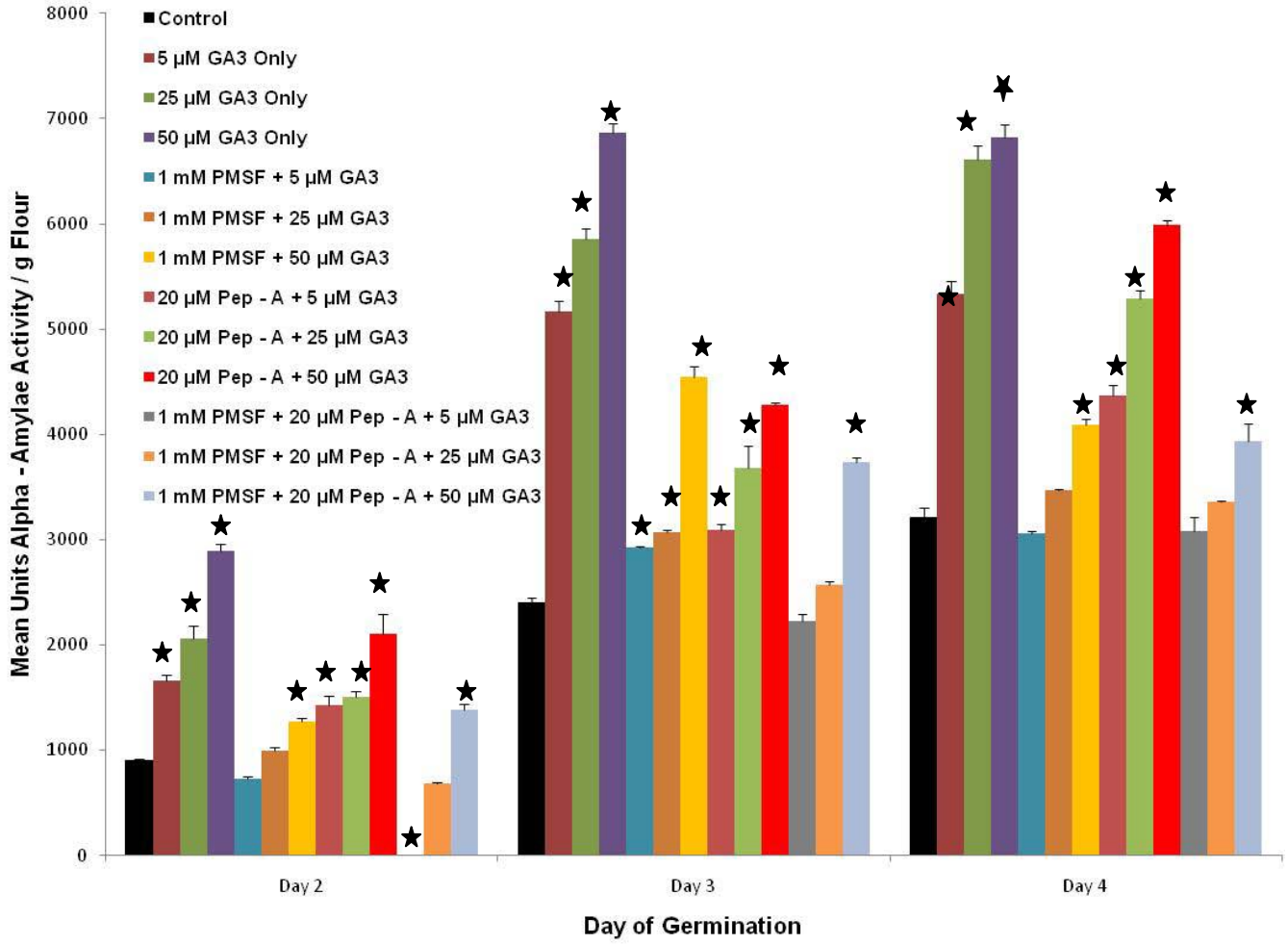


Figure 3.53: Effect of the addition of 5 µM, 25 µM and 50 µM GA_3 alone and in combination with 1mM PMSF and 20 µM pepstatin A, and 1 mM PMSF and 20 µM pepstatin A together on subsequent α – amylase activity. The addition of 5 µM, 25 µM and 50 µM GA_3 alone to the barley grain germination media was shown by two – way ANOVA and Bonferroni post hoc analysis to produce a significant increase in the activity of α – amylase at each day of germination investigated. The addition of the different GA_3 concentrations alongside 1 mM PMSF and 20 µM pepstatin A and both the inhibitors together produced a GA_3 concentration dependent increase in the levels of α – amylase activity. Error bars represent standard deviation, n = 3, ★ = Significant differences ($p = < 0.05$) in α - amylase activity between the control and inhibitor on that particular day of germination

3.5.3: β Amylase

The effects of the four protease class – specific inhibitors on the activities of β - amylase was investigated using germination studies, four day kilned malt and pure, commercially acquired, β – amylase.

Preliminary β – amylase activity studies were carried out using four day malt that had been kilned. Since β – amylase is present in an inactive form within the grain bound to a proteinaceous inhibitor molecule the commercial assay kit required an extraction step. Two – way ANOVA and Bonferroni post hoc analysis of the data in Figs. 3.54 and 3.55 show that there was little difference in the activity of β – amylase in the presence of E – 64 and 1, 10 phenanthroline (cysteine and metalloprotease inhibitors respectively) when they are added at the extraction stage, but that in the presence of pepstatin A (class specific aspartate protease inhibitor) there was a significant decrease in the levels of β – amylase activity observed (Fig. 3.54). Furthermore, statistical analysis showed that in the presence of PMSF (the class specific serine protease inhibitor) there was a significant increase in the levels of β – amylase activity when added at the extraction stage (Fig. 3.54).

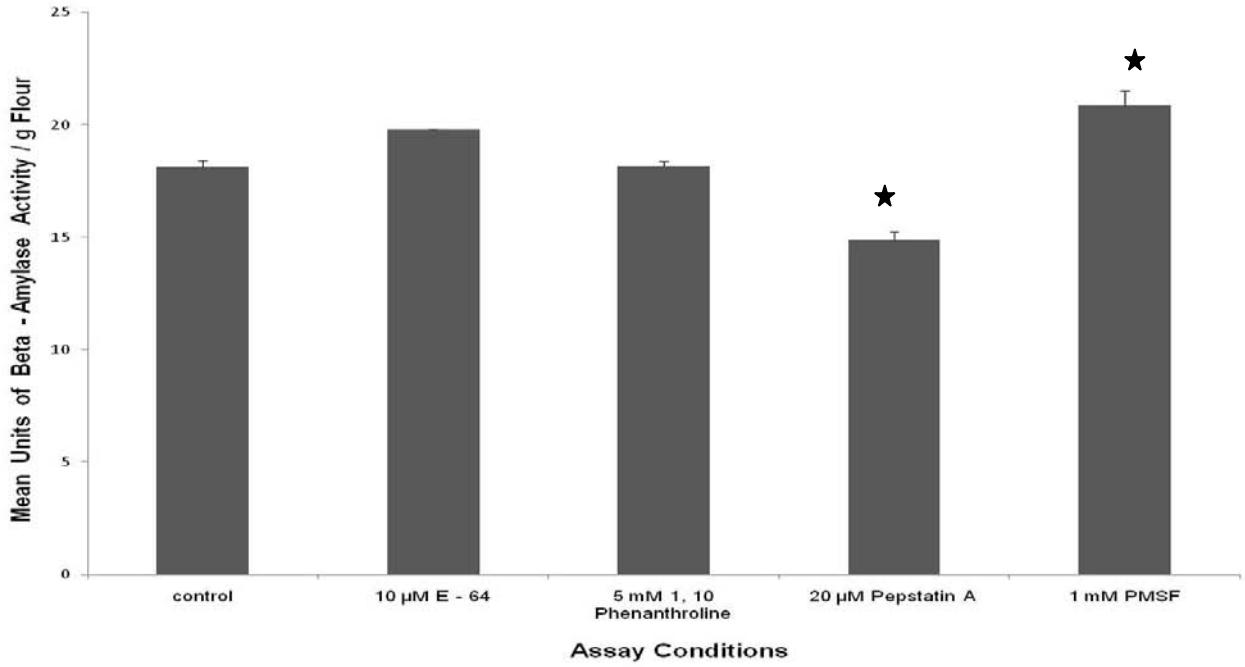


Figure 3.54: Effects of the addition of class specific protease inhibitors at the extraction stage of the β – amylase assay using four day kilned malt. The addition of 20 µM pepstatin A was shown by two – way ANOVA and Bonferroni post hoc analysis to produce a significant decrease in the levels of β – amylase activity, whereas the inclusion of 1 mM PMSF produced a significant increase in the levels of β – amylase activity. ★ = significant differences ($p = < 0.05$). Error bars represent standard deviation, n = 3

When the class specific protease inhibitors were added at the assay stage (at which time β – amylase should already be released from its inhibitor) a different picture emerged (Fig. 3.55) as there was a significant decrease in the levels of β – amylase activity in the presence of the class specific metalloprotease inhibitor 1, 10 phenanthroline. Since this inhibition was observed at the assay stage and not at the extraction stage it is most likely that the decrease is a result of the chelation of divalent cations and not the inhibition of metalloproteases as β – amylase should have already been activated during the extraction stage.

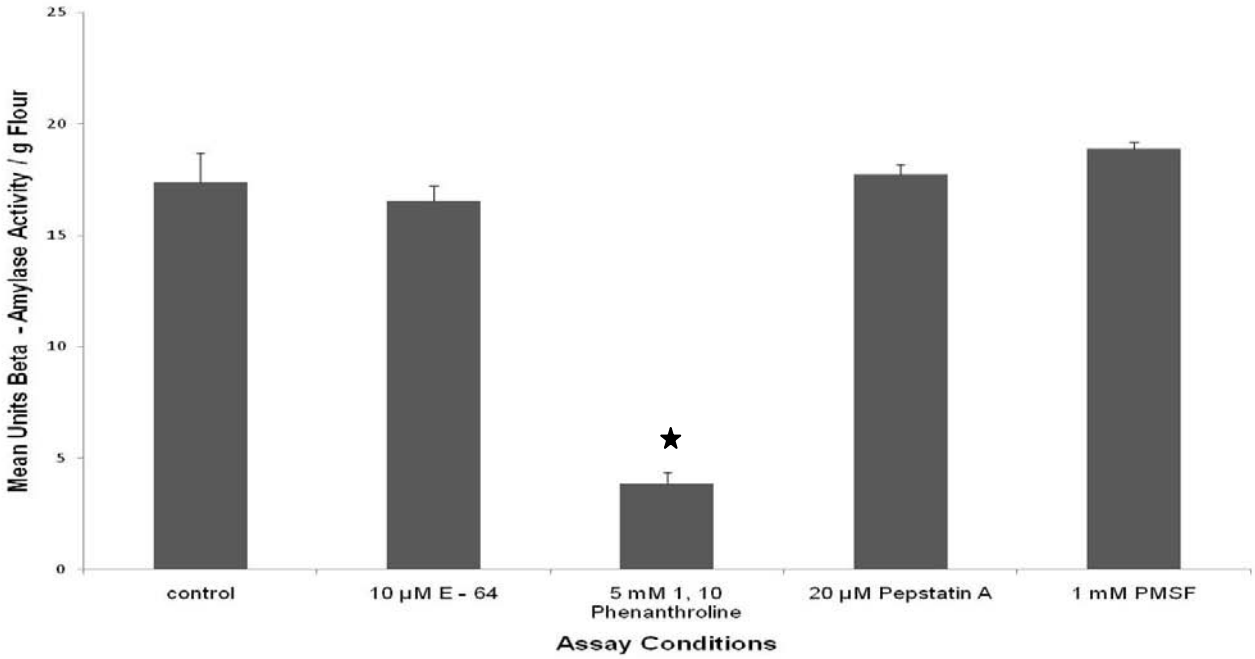


Figure 3.55: Effects of class specific protease inhibitors on the activity of β – amylase from four day kilned malt when added at the assay stage. Two – way ANOVA and Bonferroni post hoc analysis showed that 1, 10 phenanthroline was the only inhibitor used to have a significant effect on the activity of β – amylase. ★ = significant differences. Error bars represent standard deviation, n = 3

The involvement of divalent cations in the activity of β - amylase was further investigated by assaying pure, commercially acquired β – amylase (Fig. 3.56) in the presence of 5 mM 1, 10 phenanthroline and the divalent cation zinc (in the form of $ZnSO_4$). This study showed that the inhibition of β – amylase activity induced by 5 mM 1, 10 phenanthroline can be partially rescued by the addition of 20 mM $ZnSO_4$ to the assays demonstrating that like α – amylase, β – amylase may also require the presence of divalent cations for its activity, but zinc may not be the optimal metal ion for activity. 1, 10 phenanthroline was solubilised in methanol to create a stock solution for use in the assays, so to check that it was indeed the 1, 10 phenanthroline that produced the inhibitory effects and not methanol, a control experiment was set up where an equivalent methanol concentration to that of the 5 mM 1, 10 phenanthroline assays was set up. These showed that the methanol concentration used had little effect on the levels of pure β – amylase activity within the assays thus confirming that it was 1, 10 phenanthroline that was mediating the inhibition of β – amylase activity.

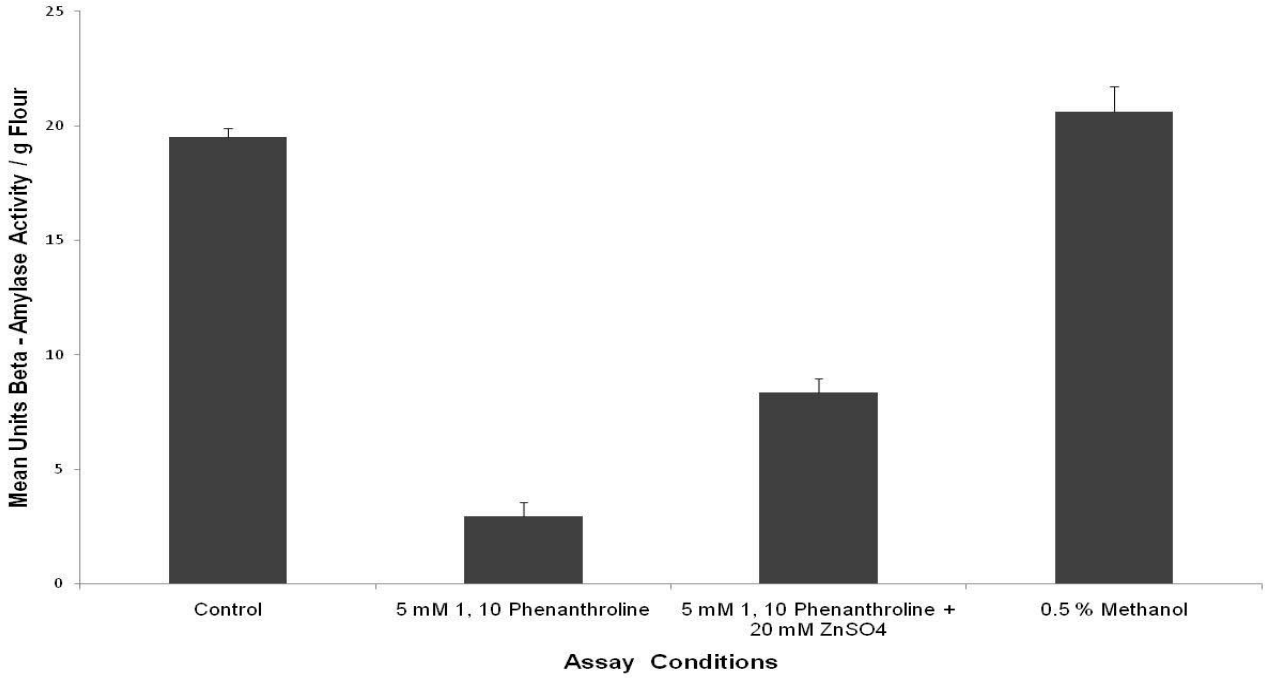


Figure 3.56: Effects of 1, 10 phenanthroline and zinc on the activity of pure β – amylase. Since 1, 10 phenanthroline was solubilised in methanol, β – amylase was assayed in the presence of methanol as a control to ensure that it was 1, 10 phenanthroline and not methanol that was inhibiting the activity of β – amylase. Error bars represent standard deviation, n = 3

Following the assays on four day kilned malt class specific protease inhibitors studies were carried out on germinating barley grains. In these experiments the four different class specific protease inhibitors were included in the germination media of barley grains and their effects on both total (i.e. all the β – amylase present in the grain whether bound or unbound) and free β – amylase (just the unbound) activity was investigated (Figs. 3.57 and 3.58).

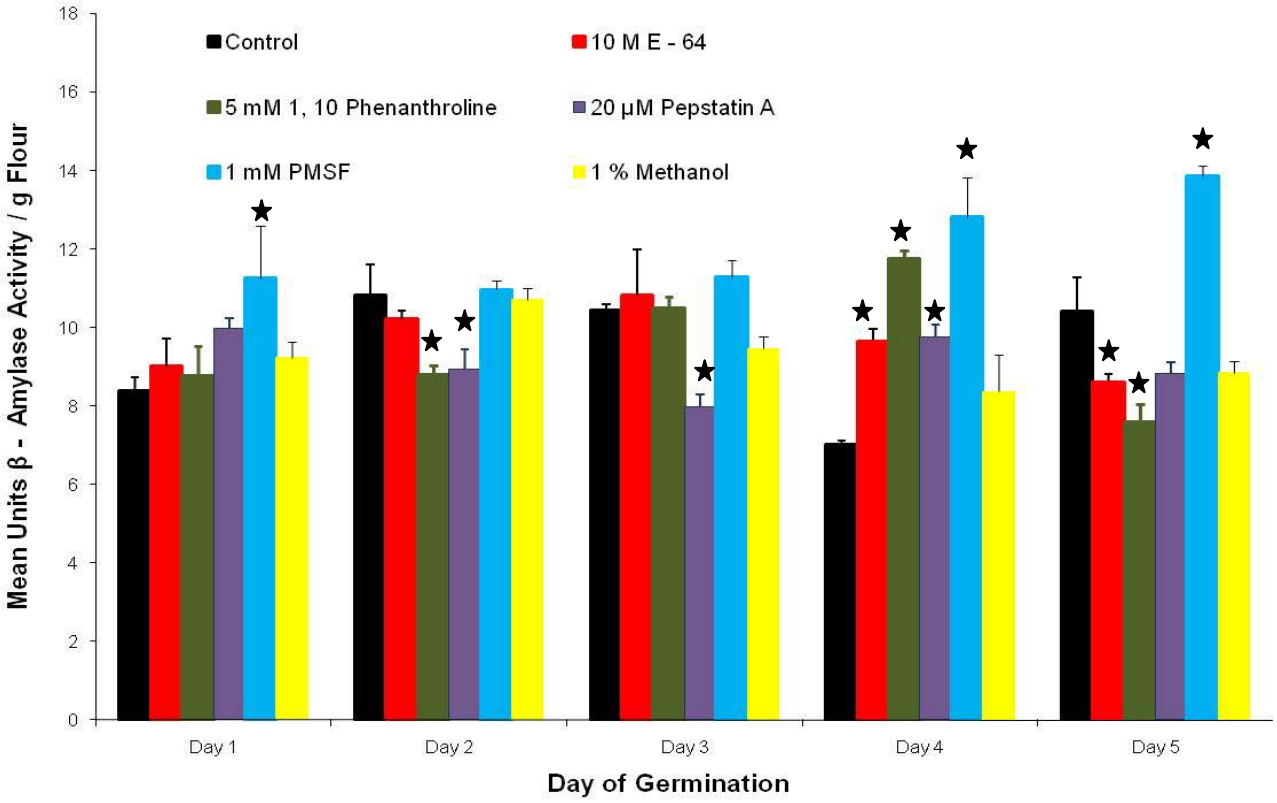


Figure 3.57: Effects of class specific protease inhibitors on the activity of total β – amylase during barley grain germination. Two – way ANOVA and Bonferroni post hoc analysis show that all four protease inhibitors significantly increased the levels of total β – amylase activity on day four of germination, and that 1 mM PMSF was the only inhibitor to significantly increase the total levels of β – amylase activity on any other germination day (days one, four and five). Error bars represent standard deviation, n = 3, ★ = Significant differences ($p = < 0.05$) in total β - amylase activity between the control and inhibitor on that particular day of germination

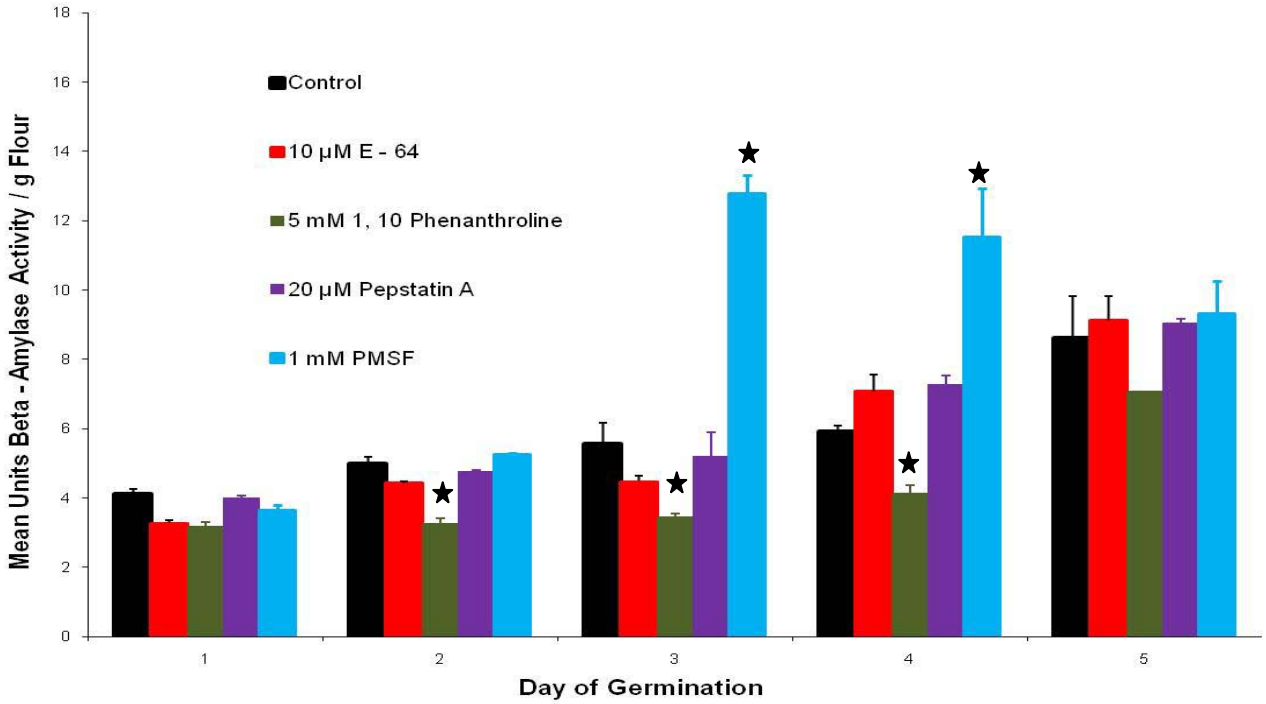


Figure 3.58: Effects of class specific protease inhibitors on the activity of free β – amylase during barley grain germination. Two – way ANOVA and Bonferroni post hoc analysis show that none of the four protease inhibitors significantly affected the levels of free β – amylase activity on days one and five of germination, and that 1 mM PMSF significantly increased the levels of free β – amylase activity at days three and four of germination, whereas 5 mM 1, 10 phenanthroline significantly decreased the levels of free β – amylase activity on days two to four of germination. Error bars represent standard deviation, n = 3, ★ = significant differences ($p = < 0.05$) in free β - amylase activity between the control and inhibitor on that particular day of germination

Two – way ANOVA and Bonferroni post hoc analysis of the data in Figs. 3.57 and 3.58 showed that the addition of 5 mM 1, 10 phenanthroline to the germination media of barley grains produced a significant decrease in the levels of both free and total β – amylase activity, significantly decreasing free β – amylase activity on days two to four of germination, and only on days two and five of germination for total β – amylase activity. These results could further demonstrate the potential role for divalent cations in the activity of β – amylase, or that there may be a requirement for divalent cations such as zinc for the release and thus activation of β – amylase during barley grain germination. However, it is also possible that the reduction in the activity of free β – amylase (Fig. 3.58) in the presence of 5 mM 1, 10 phenanthroline could be a result of a decrease in the protease synthesis resulting from the inhibition of germination, thus resulting in less β – amylase being activated.

On germination day four, the total β – amylase activity data showed a decrease in the control values compared to the other germination days and a significant increase in β – amylase activity levels in the presence of all four protease inhibitors (Fig. 3.57). This result could be interpreted as indicating a role for all four protease classes in the activity of β – amylase specifically for day four of the germination process however, since the control levels are significantly lower than on any other germination day, and that the addition of 5 mM 1, 10 phenanthroline decreased total β – amylase activity on all other germination days these observations are probably most likely to be a result of aberrantly low control values rather than of any special role at day four for the different protease classes.

The class specific protease inhibitors E – 64 and pepstatin – A were shown to have no significant effects on the activity of free β – amylase during the germination studies indicating that neither cysteine nor aspartate proteases have a role in free β – amylase activity (Fig. 3.55). However as Fig. 54 shows, both E – 64 and pepstatin – A have significant inhibitory effects on total β – amylase activity on day five and days two and five respectively. This data could demonstrate a role for the cysteine and aspartate class proteases in the positive regulation of β – amylase protein amounts within the grain or in its activation, possibly though the degradation of β – amylase inhibitors. However, since these observations were not seen in the free β – amylase activity assays further studies would be required to obtain a clearer picture of the roles of these two protease classes in β – amylase activity during grain germination.

In both the total and free β – amylase activity assays the addition of up to 1 mM PMSF to the germination media produced a significant increase in β – amylase activity levels at days one four and five, and days three and four respectively (Figs. 3.57 and 3.58). In view of these striking results, the experiments on the effects of PMSF on free β – amylase were repeated but using higher levels of PMSF (Fig. 3.59), and the data shows significantly enhanced free β – amylase activity in treated grains. These results indicate a role for serine class proteases in the negative regulation of β – amylase activity possibly via β – amylase degradation or the possible regulation of some aspect of β – amylase activity during the germination process. These results support findings by Schmidt & Marinac (2008) that

serine proteases degrade β – amylase during the later stages of germination possibly in a potential amino acid scavenging role.

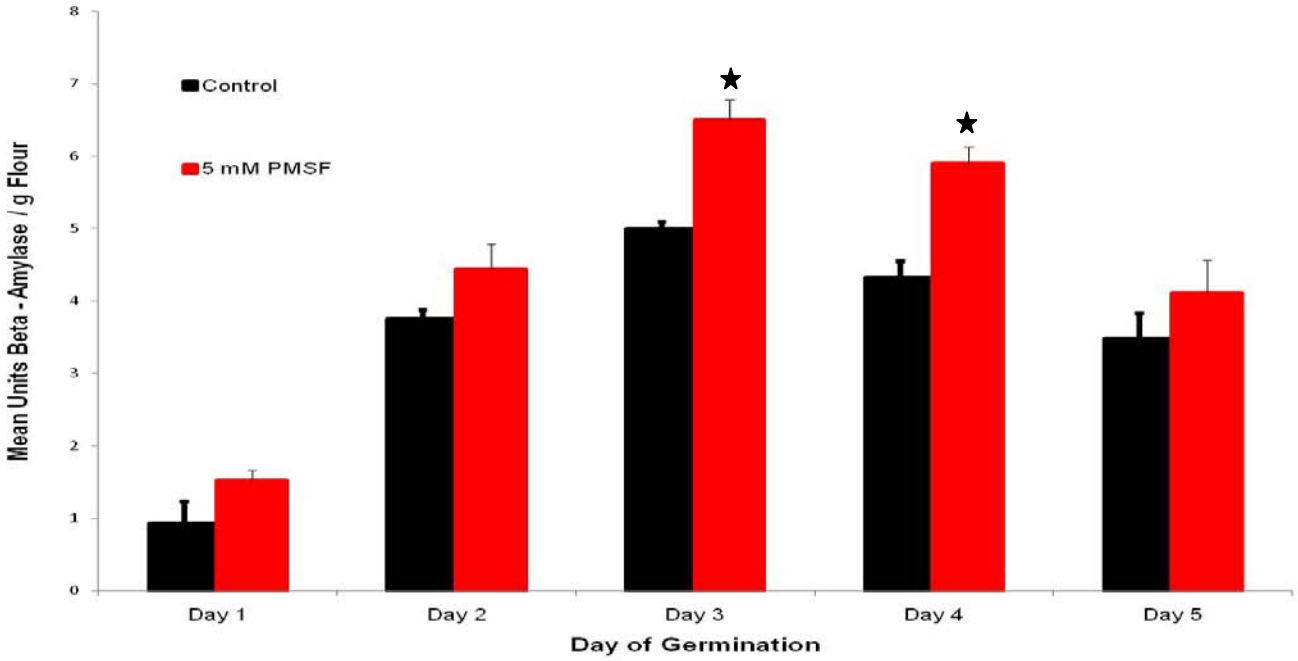


Figure 3.59: Effect of 5 mM PMSF on the activity of free β – amylase during barley grain germination**.** Two – way ANOVA and Bonferroni post hoc analysis showed that the addition of 5 mM PMSF significantly increased the levels of β – amylase activity at days three and four of germination. Error bars represent standard deviation, n = 3, ★ = significant differences (p = < 0.05)

3.6: Serine Protease Enrichment

The potential of serine proteases to degrade β – amylase formed the basis of the serine protease enrichment process whereby a new extraction technique (using grains “cracked” in a coffee grinder and germinated in an extraction buffer in the presence of gibberellic acid – section 2.5.1) was employed in an attempt to identify serine proteases potentially involved in β – amylase degradation.

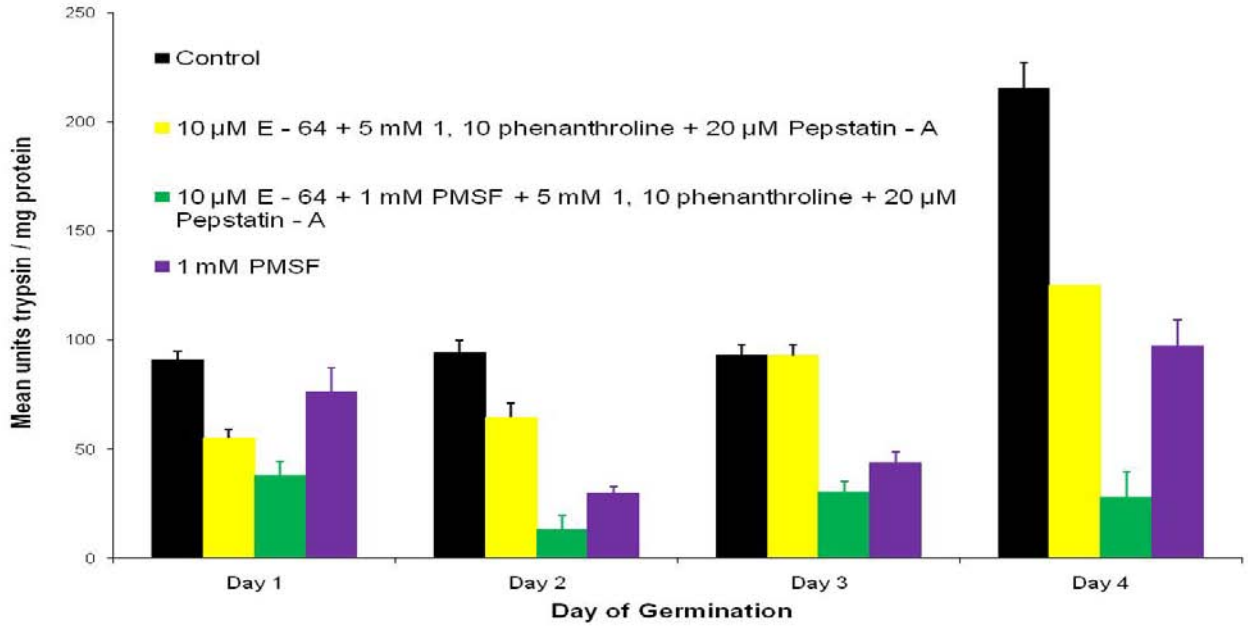


Figure 3.60: Effects of different protease inhibitor combinations on the levels of protease activity excreted into the medium at days one to four of extraction. Results showed that day three exhibited the highest levels of serine protease activity. Error bars represent standard deviation, n = 3

The supernatant was collected every 24 hours over four days and the effects of different protease inhibitor combinations on each extract day was investigated in an attempt to ascertain which extract day contained the highest levels of serine class protease activity (Fig. 3.60). These investigations showed that the extract collected on day three exhibited the highest levels of serine protease activity (Fig. 3.60), thus day three was selected for further investigation.

Fig. 3.61 shows the effects of assaying pure β – amylase in the presence of different volumes of day three extract and extraction buffer. The day three extract produced a decrease in β – amylase activity and the more day three extract added, the greater the inhibitory effect. Thus day three was selected to be the extract day investigated.

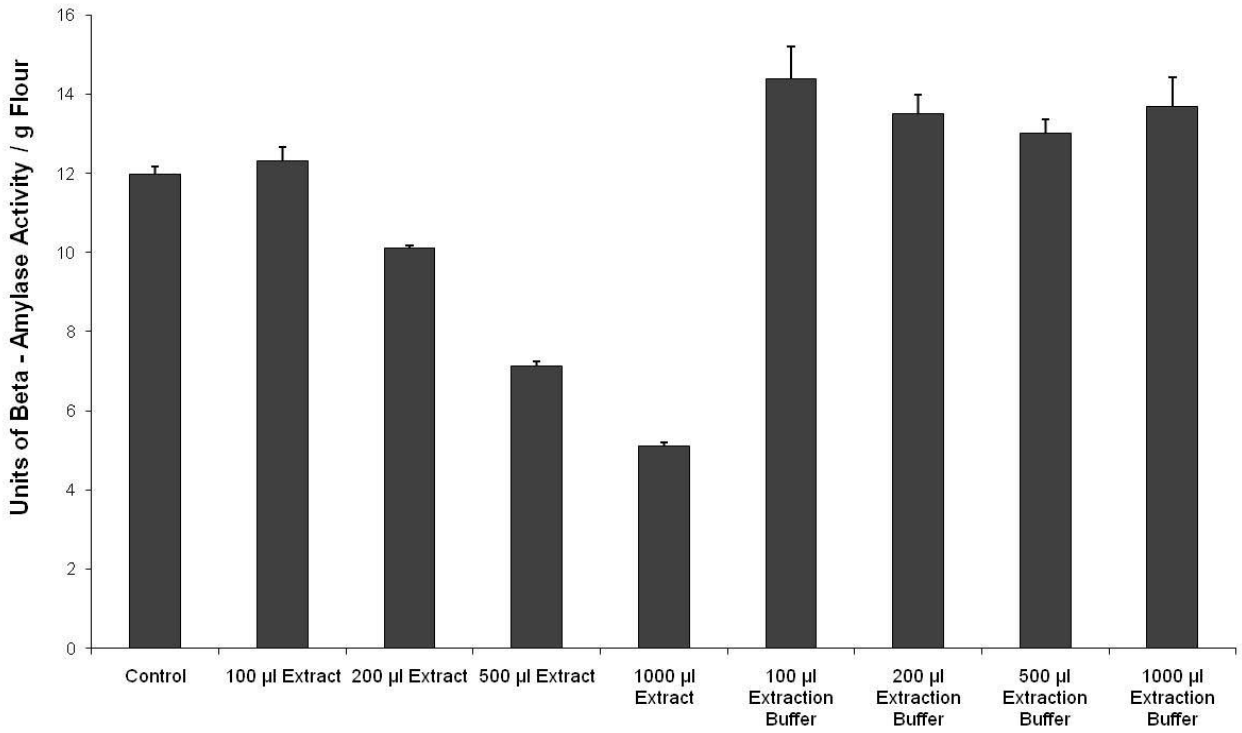


Figure 3.61: Effects of varying amounts of day three barley extract on the activity of pure β – amylase. The more extract added to the purified β – amylase the lower the β – amylase activity, this decrease was not observed when extraction buffer alone was added. Error bars represent standard deviation, n = 3

Day three extract was fractionated at pH 5 on an SP anion exchange column (section 2.5.3) in the presence of BSA and E – 64 to inhibit autolysis of any proteases within the extract. Fractions were then assayed in the presence and absence of 1 mM PMSF (Fig. 3.62) and those fractions exhibiting serine protease activity were further analysed by SDS – PAGE (Fig. 3.63) and bands were selected for MALDI-TOF mass spectroscopic analysis. However, only one positive protein identification could be made which was a Z – type serpin (molecular weight = 43307, MASCOT significance value E = 0.00067, 22 % sequence coverage). These proteins are known to be inhibitors of serine class proteases ((Hejgaard et al, 1985).

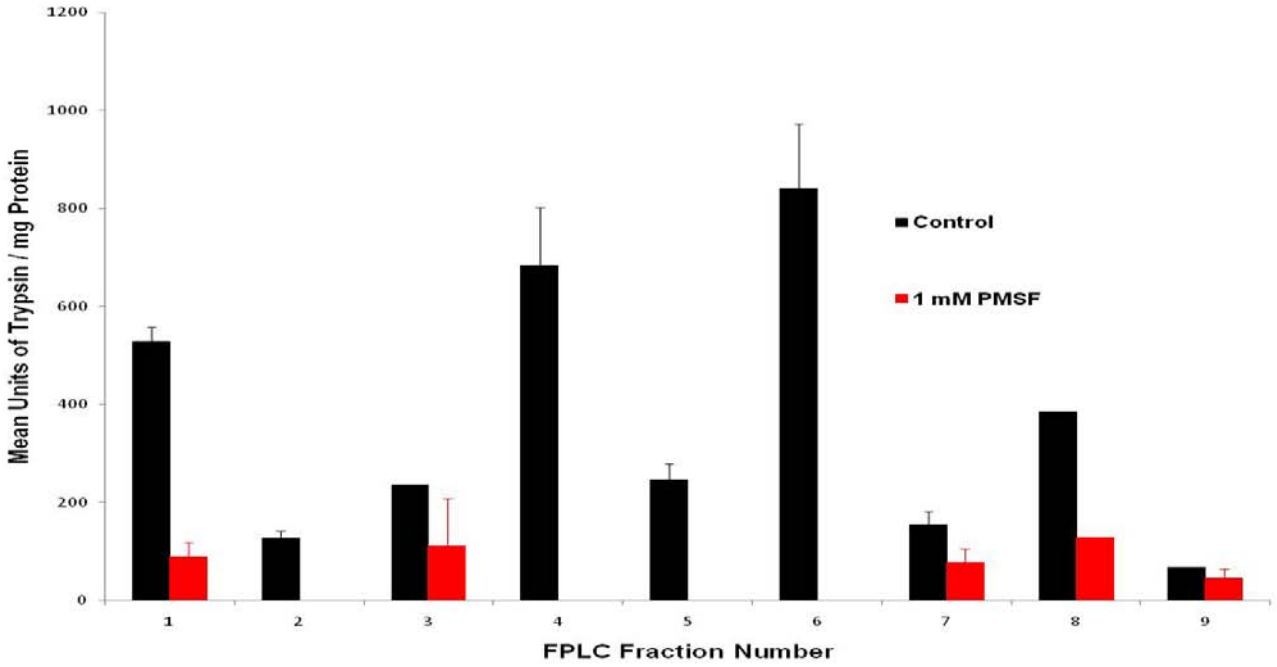


Figure 3.62: Azocasein protease activity assay of the fractions from the SP anion exchange fractionation of day three extract. Fractions four to six were shown to contain the highest levels of serine protease activity, thus these were selected for SDS – PAGE analysis. Error bars represent standard deviation, n = 3.

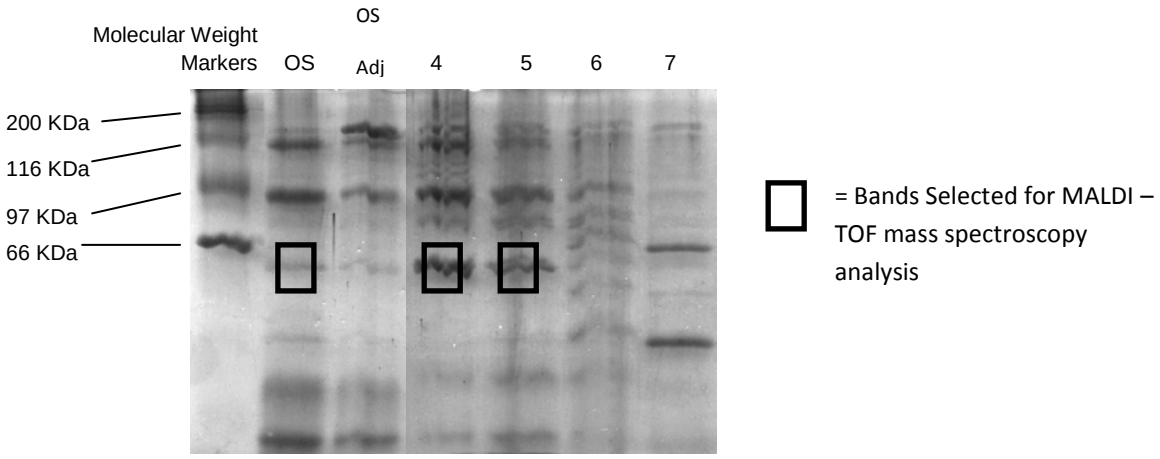


Figure 3.63: SDS – PAGE analysis of the serine protease activity containing fractions from the SP anion exchange fractionation of three day extract. "OS" stands for original sample (the three day extract) and "OS Adj" stands for the three day extract after the addition of BSA and 10 µM E – 64. The numbers represent those fractions which displayed serine protease activity.

Much of the previous research into the protease enzymes of barley grains has focused upon their roles in the mobilisation of the grains protein stores during malting and germination (Jones, 2005). However, these are not the only possible functions of the barley grain proteases as they have been shown to be involved in the regulation of other enzyme classes, such as β - amylase (Schmitt & Marinac, 2008). Moreover, by virtue of their function (that is to catalyse the breakdown of proteins) proteases fulfil important roles as major biological regulators in nearly all biological systems (Schilling & Overall, 2007). In all higher organisms, the chief proteolytic pathway for protein turnover is the ubiquitin / 26S proteasome pathway (UbS) (Smalle & Vierstra, 2004) which functions through the ubiquitination of target proteins to degrade such a large range of intracellular proteins that there are very few plant biological processes that do not involve this proteolytic pathway in some way (Sullivan et al, 2003). Furthermore, through its involvement in plant hormonal signalling, the UbS plays a central role in nearly every part of plant growth and development including responses to light, flower development, germination, stem elongation and defence against pathogen invasion (Wang & Deng, 2011). One of the best defined roles for the UbS system is in GA signalling where UbS has been shown to be a positive regulator, as when GA binds to its receptor, the UbS system, through associations with the GA receptor, is activated which induces the ubiquitination and subsequent degradation of the DELLA proteins thus removing their inhibition of GA – responsive genes. With these diverse and wide ranging roles of protease enzymes in mind it could be postulated that the proteases present within the germinating barley grain may perform similar functions. To investigate this, this study aimed to use proteomic and enzyme assay based techniques to characterise the different protease classes present in barley grains during malting and germination, and also to investigate their roles in the malting and germination processes.

4.1: Protease Assay Selection

The choice of an appropriate assay is very important in any enzymatic investigation as the assay used should be based upon a substrate whose hydrolysis products can be easily measured, has a structure that is readily digestible by the enzyme or enzymes under investigation and be performed under assay conditions that are as representative as possible of the environment in which catalysis would take place *in vivo* (Jones. 2005). These selection criteria lead to the use and adaptation of the azocasein protease assay as described by Jones et al, 1998 as azocasein has an easily quantified hydrolysis product because it releases the azo dye upon hydrolysis and the intensity of the dye released is directly proportional to the amount of protease activity present in the grain and can be

measured spectrophotometrically at a UV wavelength of 440 nm. Azocasein was shown in protease assay trials conducted during this study to be more reproducible than assays using other substrates such as azogelatin used by Jones et al in 1998 (results not shown). Furthermore, azocasein was chosen over purified barley grain storage proteins such as hordeins because these are not easily available commercially, and would have to be derivatised prior to use. In addition little information is available on the endogenous targets of barley grain proteases, for example it is not known whether all barley grain proteases would degrade these storage proteins as it has been previously reported that some aspartate class proteases are unable to degrade hordein (Jones, 2005). For these reasons, and as it is known to be digested by a wide range of proteases, azocasein was selected as a substrate. In addition, azocasein has been used successfully in previous studies in the discovery and purification of two cysteine proteases, EP – A and EP – B (Koehler & Ho, 1988 and Koehler & Ho, 1990).

4.2: Kilned Malt Compared to Green Malt

In this study both kilned and green malt were employed. Green malt was used firstly in order to preserve as much of the enzyme activity as would be present in malting grains as possible. However, because green malt is still germinating it could not be stored on the bench and was thus frozen in aliquots at – 20 ^{o}C until required. When the grains were repeatedly defrosted their measured protease activity levels were non - reproducible, thus the decision was made to move to the use of malt kilned at a low temperature (60 ^{o}C). Previous studies have shown that kilning at temperatures of up to 85 ^{o}C had little degradatory effect on the activities of barley grain proteases (Jones et al, 2000) and that some cysteine protease activities were enhanced by the kilning process. The possible reasons for this enhancement could be that some proteases may undergo a conformational change and thus become active at higher temperatures, or that protease – inhibitor complexes may be broken down at higher temperatures thus liberating free and therefore active protease enzymes. In this study the kilning temperatures were kept low (i.e. to 60 ^{o}C) as despite the proteases activities reportedly not being degraded by the kilning process it was important to maintain as much protease stability as possible.

4.3: Class Specific Inhibitor Studies

The azocasein protease assay was used, in combination with different class specific protease inhibitors and the reducing agent DTT, to investigate the contributions of the members of the four different protease classes to the overall levels of proteolysis, in green malt, at days two and four of the malting process. Days two and four micro malted grains were selected for this study because the levels of protease activity are known to increase as germination progresses (Zhang & Jones, 1995) and that there are also reported to be temporal variation in the expression and activities between the different protease classes, thus a comparison of days two and four micro malted malt was chosen to compare the protease activities in early and later stages of malting.

DTT is a reducing agent and is known to activate cysteine proteases (Jones et al, 1998; Beynon & Boyd 2001). When 5 mM DTT was added to protease assays at different pH values the largest increases in protease activity compared to control values were observed at pH four to six in both day two and day four malt (Figs. 3.3 and 3.4), with day two malt showing a slightly lower level of protease activation. These increases in the levels of protease activity in the presence of DTT have also been shown in mash sample and green malt crude extract studies (Jones and Budde, 2003). Since cysteine proteases are known to be activated by reducing conditions (Jones et al, 1998; Beynon & Bond, 2001) it is highly probable that the increases in protease activity observed after addition of DTT are as a result of increases in cysteine protease activity. Furthermore, due to the slightly larger increase in protease activity at day four than at day two (protease activity, in the presence of DTT at pH 5.0 was approximately four times greater than the control values on day four, compared to only approximately three times higher at day two) these results could indicate that the cysteine class proteases are more active, or present in greater amounts, at day four of malting than at day two. However, when the effects of the class specific cysteine protease inhibitor, E – 64 on the levels of protease activity in days two and four malt were investigated the largest amount of inhibition of protease activity was observed at day two (Figs. 3.5 and 3.6) indicating that the cysteine proteases could be either more active or present at a higher level at day two of malting than they are at day four. The possible reasons for these differing results could be that the control values for the DTT studies were falsely low (as they were lower than any of the other activator / inhibitor study control values (Figs. 3.3 to 3.12)) thus the extent of the increase in protease activity in the presence of DTT could have been exaggerated. Furthermore, reducing conditions may not only activate cysteine proteases, it could be that the activities of other protease classes are enhanced by the presence of

reducing agents such as DTT, however further research would be required to fully elucidate the reasons for these differing results.

To further characterise the activities of the four different protease classes protease assays were carried out at a range of pH values. At days two and four, and for all four protease classes the optimum pH (i.e. the pH at which the greatest amount of inhibition of protease activity was observed in the presence of the different class specific protease inhibitors) was found to be in the range of pH four to six (Figs. 3.4 to 3.12) and as a result they may all be active within the same cellular or grain compartment. For example, it has been shown that during germination GA induces the acidification of vacuoles located within the aleurone layer (Swanson & Jones, 1996), thus it could be postulated that members of all four proteases are present within these acidified vacuoles. One notable exception is the metalloproteases because at both days two and four of micromalting (Figs. 3.7 and 3.8) the inclusion of 1, 10 phenanthroline into the assay media resulted in inhibition across the pH scale of the investigation indicating the possibility of metalloproteases being active in different parts of the barley grain or in different cellular compartments to the other proteases classes. These results agree in part, with those of Zhang & Jones (1995) where through the use of 2D zymograms of four day germinated green malt extracts and the incubation of these gels in different buffer pHs following separation, they ascertained that the pH optima of the cysteine, aspartic and metallo class protease activities identified were in the region of pH three to pH six. However, they also found that the pH optima of the serine class protease activities identified in their study lay at pH eight and above, a result that contradicts those of the current study. A possible explanation for this could that they used 2D zymograms to detect the protease activities whereas the present study used liquid assays and the effects of the electrophoresis conditions might have influenced the properties of the enzymes in Zhang & Jones' extracts. Also, the differences could be a reflection of the different extraction techniques employed as Zhang & Jones extracted in the presence of cysteine and also EDTA, whereas neither agent was added to the extract buffers in the current study. It is possible that the alkaline serine proteases were more stable in the presence of reducing agents such as cysteine. Furthermore, the differences in serine protease pH optima could also be due to varietal differences between the two studies as Zhang & Jones used Morex barley while the present study used Oxbridge barley.

When the percentage inhibition of protease activity in the presence of each of the inhibitors at each day of micromalting and at each pH was calculated and added together a figure higher than 100 % was obtained in both the day two and day four micro malted grains. This result is probably most likely to be caused by the cross – inhibition of members of different protease classes by particular protease inhibitors. For example, PMSF is known to inhibit some cysteine proteases as well as the serine class proteases. Furthermore, 1, 10 phenanthroline exerts its inhibitory effects through the chelation of divalent cations, thus it may be possible that 1, 10 phenanthroline could also inhibit the activity of members of the other proteases classes that may rely on divalent cations for their activity. For example the serine protease thiocalsin is known to require Ca^{2+} ions for its activity (Besse et al, 1996). Furthermore, it is possible that a member or members of one protease class may regulate another protease class through degradation of the protease or protease inhibitors, thus when the inhibitory protease class is inhibited the activities of their target proteases increase. The net result all of these potential factors could be that the protease activity levels present under the influence of different class specific protease inhibitors may not be completely representative of the contribution of that specific protease class to the overall levels of protease activity as members of another protease class may also be inhibited, or that some of the protease activity knocked out by the inhibitor may be replaced by that of another protease class.

4.3.1: Germination Studies

When Oxbridge barley grains were germinated in the presence of the different class specific protease inhibitors (Fig. 3.24) a slightly different picture emerged from that of the two and four day malt crude extract studies, because whereas E – 64 produced the greatest amount of inhibition of protease activity in the crude extract studies, here 1, 10 phenanthroline exerted the greatest effect on protease activity by being the only inhibitor that produced a significant decrease in the levels of protease activity at all five days of germination (Fig. 3.24). Germinating in the presence of E – 64 and PMSF also resulted in a significant decrease in the overall levels of protease activity (Fig. 3.24) but only on days four and five respectively. The differences between these results and those of the two and four day micro malted barley studies could be due to the inhibitors having other physiological effects on the grain. This is indicated by the inhibition of grain germination in the presence of 1, 10 phenanthroline, thus germination – dependent events such as protease gene expression and activation may also have been inhibited, resulting in a decrease in protease activity levels during germination. Furthermore, it is known that PMSF in particular has a short half

life in aqueous solution (just under two hours at pH 7 at 25 ^{o}C (James, 1978)) thus it could be that the effects of inhibitors such as PMSF are limited compared to their effects when they are added directly to the assay media. Also, it could be argued that it is not valid to directly compare the results obtained from malt extracts with those from extracts of germinated grains as the malted grains are steeped before they are germinated so it is possible that some of the metabolic activities of early germination may have taken place earlier in the malting grains than in the germinating grains however, since this difference is only temporal, as steeping is most probably equivalent to day one of germination, and thus does not have an effect on the processes of germination these effects are limited.

Observations were also made on the effects of the four different class specific protease inhibitors on barley grain germination, shoot emergence and rootlet elongation (i.e. average rootlet length). In these studies 1, 10 phenanthroline was the only inhibitor to have a large effect on all three parameters (Figs. 3.21 to 3.23), with PMSF having an effect on shoot emergence and rootlet elongation, and E – 64 and pepstatin – A only having a slight effect on shoot emergence. These results indicate that the different protease classes have diverse roles in barley grain germination. Elucidating the importance and roles of the metalloproteases in these germination processes is however, not straight forward, as the inhibition of germination by the 1, 10 phenanthroline mediated chelation of divalent cations is most likely to be the reason for the low levels of shoot emergence and rootlet elongation observed in these grains, as divalent cations such as iron, zinc and manganese are known to be essential to the growth and development (including germination) of plants (Palmer & Guerinot, 2009). For example, manganese is known to be essential for the activation of enzymes involved in the GA biosynthetic pathway (Hänsch & Mendel, 2009) and for the functioning of antioxidant enzymes and for the efficient functioning of aerobic respiration (Alscher et al, 2002), thus the chelation of manganese ions by 1, 10 phenanthroline could go towards explaining the reduction in overall protease activity levels and the inhibition of germination observed. Furthermore, it has been shown that the growth of barley cultivars with high efficiency for manganese uptake and remobilisation, in manganese deficient soils (that is in soils where manganese, although present, is bound in a form which has low phytoavailability) show both improved growth and increased crop yield compared to manganese inefficient barley plants (Pallotta et al, 2000).

Furthermore, *Arabidopsis* studies involving the double knockout of the vacuolar iron transporters AtNRAMP3 and AtNRAMP4 have shown that under iron limiting conditions, rootlet growth during seed germination was inhibited during the first four days of germination, an effect which could be rescued by the expression of either transporter in the double knockout plants (Lanquar et al, 2005), demonstrating the importance of iron in the process of rootlet growth and supporting the hypothesis that it is the chelation of divalent cations that is primarily responsible for the effects of 1, 10 phenanthroline on germination. Furthermore, it has been shown in *Acer pseudoplatanus. L* that iron is important for the function of the electron transport chain in the mitochondria and that iron limiting conditions impair the synthesis of ATP (Pascal & Douce, 1993). Thus it could be that the chelation of iron by 1, 10 phenanthroline results in an impairment of the electron transport chain and ATP synthesis resulting in less energy being available within the seeds for germination and its related processes. Thus further investigations are required into the specific involvements of the different divalent cations in the germination process. Also, it would be interesting to grow plants to maturity in the presence of 1, 10 phenanthroline, and 1, 10 phenanthroline plus different divalent cations to observe what effects these growth conditions may have on overall barley plant growth and development.

Taken together, the two and four day micro malted crude extract assays and the germination studies point to differences in not only the temporal activities of the four protease classes, a result that is supported by existing literature (Wrobel & Jones, 1992, Zhang & Jones 1995 and Jones, 2005), but also the diversity of the potential roles and processes that the four protease classes may fulfil in overall grain germination physiology. Due to the breadth of biochemical processes covered in these germination studies there is a large body of potential work that could emerge. For example, similar studies could be carried out on other barley varieties (as only Oxbridge was used here) and also in wheat, rice and maize to investigate whether the results shown here are particular to Oxbridge or whether they are more universal throughout barley varieties and cereals in general. Also, the involvement of the different protease classes in the determination of “good” and “poor” malting varieties could be explored by repeating these studies using animal feed standard grains.

4.3.2: Germination Studies - Divalent Cations, 1, 10 Phenanthroline & Barley Grain Germination

As previously mentioned, grains germinated in the presence of the divalent cation chelator 1, 10 phenanthroline showed reduced germination (Fig. 3.21). These grains also showed reduced rootlet elongation (Fig. 3.23) and shoot emergence (Fig. 3.22). 1, 10 phenanthroline is a class specific metalloprotease inhibitor and it exerts its inhibitory effects through the chelation of divalent cations. It is probable that the inhibitory effects of 1, 10 phenanthroline observed here were brought about by the chelation of divalent cations. To further investigate the potential significance of divalent cations in the germination process germination rescue experiments were carried out. These experiments consisted of germinating barley grains in the presence of both 1, 10 phenanthroline and different divalent cations (Figs. 3.25 to 3.46). The results of these investigations showed that the inclusion of specific divalent cations in the germination media along with 1, 10 phenanthroline rescued germination, rootlet elongation and shoot emergence. However, these experiments showed that it was not just the presence of divalent cations in general that rescued the 1, 10 phenanthroline mediated inhibition of germination, but specifically the transition metal ions as observed in the experiments where the inclusion, along with 1, 10 phenanthroline, of the transition metal ions Zn^{2+}, Mn^{2+} and Fe^{2+} resulted in germination rescue (Figs. 3.25, 3.27 and 3.39), but the inclusion of the non – transition metal ions Ca^{2+} and Mg^{2+} failed to rescue germination (Figs. 3.33 and 3.39). This transition metal ion mediated rescue of germination was stochiometric as a transition metal ion concentration 5 mM was enough to rescue the 5 mM 1, 10 phenanthroline mediated inhibition of germination (Figs. 3.25, 3.27 and 3.39).

The extent of germination rescue and also its timing varied with transition metal investigated as the inclusion of 5 mM and 10 mM $ZnSO_4$ rescued germination levels to the control values on day one of germination (Fig. 3.25), whereas the inclusion of 5 mM and 10 mM $FeSO_4$ did not fully rescue germination levels until day three of germination (Fig. 3.27). Furthermore, co – germinating barley grains in the presence of 5 mM $MnSO_4$ did not completely rescue germination levels to control values achieving about 97 % of the control levels by day five of germination (Fig. 3.39). These differences in the extent and timing of germination rescue by the different transition metal ions indicates variation in the grain's requirements for these ions and that possibly zinc and iron (II) are more effective at rescuing the onset of germination than manganese. Alternatively, these differences may reflect variation in the affinity of 1, 10 phenanthroline for the different metal ions, as inclusion of an exogenous divalent metal ion together with 1, 10 phenanthroline will result in the exogenously applied

metal ion out competing the chelation of the endogenous metal ions, thus 1, 10 phenanthroline may have a higher affinity for zinc and iron than it does for manganese for example.

Mineral micronutrients such as the metal ions Zn^{2+}, Fe^{2+} (and also Fe^{3+}) and Mn^{2+} are known to be essential components of the metabolic and cellular processes of all organisms including plants (Hänsch & Mendel, 2009). Moreover, Fe^{2+} is an important redox agent within plants (Palmer & Guerinot, 2009) and acts as a key component in the redox regulation of both the ethylene and GA (Hänsch & Mendel, 2009) biosynthetic pathways, thus the results here showing the rescue of 1, 10 phenanthroline mediated germination inhibition by the inclusion of $FeSO_4$ into the germination media could be a reflection of these roles as both the redox state of the grain and grain compartments, and the production of ethylene and GA are important components of grain germination. Mn^{2+} is also known to be involved in the synthesis of GA (Hänsch & Mendel, 2009) thus it could be that the inclusion of Mn^{2+} along with 1, 10 phenanthroline could fulfil similar roles to that of Fe^{2+}. In addition, Zn^{2+} is an essential plant micronutrient as it has roles in the stability and activity of many plant proteins and enzymes especially those involved in DNA – transcription, the processing of RNA transcripts and the translation of the mRNA (Hänsch & Mendel, 2009), thus it is possible that Zn^{2+} may be able to reverse the 1, 10 phenanthroline mediated germination inhibition though its roles in these processes which are essential to germination.

Observations were also made as to the effects of the different divalent cations investigated on the 1, 10 phenanthroline mediated inhibition of rootlet elongation (through average rootlet length measurements) and on shoot emergence levels from the grains. These studies showed that although the inclusion of transition metals into the germination media rescued the 1, 10 phenanthroline mediated inhibition of germination (measured as chitting) they did not necessarily rescue rootlet elongation or shoot emergence. For example, In the case of shoot emergence, none of the three $ZnSO_4$ concentrations used rescued shoot emergence back to the levels seen in control grains (Fig. 3.26), with 5 mM $ZnSO_4$ rescuing the most, achieving (from about day three of germination) approximately 80 % to 90 % of control shoot emergence levels. In the presence of 5 mM 1, 10 phenanthroline and 10 mM $ZnSO_4$ the shoot emergence levels were not rescued until day five of germination (Fig. 3.26) suggesting that despite zinc being shown to be an essential component of the onset and maintenance of barley grain germination, it is not as important for germination processes such as shoot

emergence and that in higher concentrations, zinc may be inhibitory for these germination processes. This conclusion is also supported by the observations made on the inclusion of the three $ZnSO_4$ concentrations in the absence of 1, 10 phenanthroline on the levels of both grain germination and shoot emergence (Figs. 3.25 and 3.26). These studies showed that neither 1 mM, 5 mM nor 10 mM $ZnSO_4$ had any effect on germination, but that they did have an inhibitory effect on shoot emergence at higher concentrations as the inclusion of 10 mM $ZnSO_4$ mirrored the shoot emergence levels of the 10 mM $ZnSO_4$ plus 1, 10 phenanthroline grains, indicating that at higher concentrations zinc may be inhibitory or toxic for shoot growth. The toxic effects of higher concentrations of heavy metals such as zinc on plant growth and development are well documented (Lefevre et al 2009; Palmer & Guerinot, 2009) so it is probable that the higher $ZnSO_4$ concentrations would indeed be inhibitory. Observations on the effects of zinc on average rootlet length were not made here so no conclusions as to any potential roles of zinc in this process can be made.

Observations were also made on the effects of $FeSO_4$ on both average rootlet length and shoot emergence during germination (Figs. 3.29 to 3.32) and showed that just like with $ZnSO_4$, the inclusion of the three $FeSO_4$ concentrations along with 1, 10 phenanthroline to the germination media of barley grains failed to rescue shoot emergence levels to those of the control grains. Furthermore, when $FeSO_4$ was added alone (Fig. 3.30), there was a $FeSO_4$ concentration dependent decrease in the levels of shoot emergence from approximately day two of germination, indicating that just like zinc, increasing iron (II) concentrations are inhibitory to shoot growth. When the effects of $FeSO_4$ on the 1, 10 phenanthroline induced inhibition of rootlet growth was examined it was shown that from day one of germination (Fig. 3.31) there was a $FeSO_4$ concentration dependent increase in the average rootlet length showing a possible role for iron (II) in the growth of barley grain rootlets during germination. However, the $FeSO_4$ mediated rescue was not total and was greater during earlier days of germination as rescue peaked at just over 90 % of the control values at day two of germination in the 10 mM $FeSO_4$ plus 5 mM 1, 10 phenanthroline grains, but by day five of germination these grains had average rootlets lengths of only approximately 50 % of the control grains (Fig. 3.31). This indicates that iron (II) is important for the onset and early stages of barley grain rootlet growth and development, but that by later germination it is not so important. This hypothesis is supported by the data in Fig. 3.32 which shows the effects of 1 mM, 5 mM and 10 mM $FeSO_4$ alone on the average rootlet length of germinating barley grains and illustrates that as germination progresses the rate of rootlet growth in the presence of the different $FeSO_4$ concentrations decreases in a

concentration dependent manner. The importance of iron in rootlet growth has been shown in *Arabidopsis* (Lanquar et al, 2005) where the double knockout of the vacuolar iron transporters AtNRAMP3 and AtNRAMP4 lead to the inhibition of rootlet growth during the initial four days of germination, a situation which can be rescued by the expression of one or both of these transporters in the mutant seeds.

The effects of manganese on the levels of shoot emergence and average rootlet length were also investigated (Figs. 3.40 and 3.41) and just like zinc and iron (II), the inclusion of 1 mM, 5 mM and 10 mM $MnSO_4$ along with 5 mM 1, 10 phenanthroline into the germination media of barley grains rescued germination but failed to fully rescue either shoot emergence levels or average rootlet lengths. The inclusion of 5 mM $MnSO_4$ along with 5 mM 1, 10 phenanthroline rescued shoot emergence levels to approximately 100 % of control values by day three of germination but this rescue rate dropped to approximately 70 % of the control values by germination day five showing that manganese may have a larger role during early germination. Furthermore, when the effects of the addition of 5 mM $MnSO_4$ along with 5 mM 1, 10 phenanthroline on average rootlet length measurements was investigated it was again shown that there were greater rescue levels at days one to three of germination than there were on days four to five (Fig. 3.41) providing further evidence for the greater involvement of manganese ions in the earlier stages of growth and development during barley grain germination. The effects of the addition of manganese alone were also investigated (Figs. 3.44 to 3.46) and show that germination and shoot emergence are less sensitive to the presence of elevated manganese ions than they are for elevated levels of zinc and iron (II) as adding 1 mM, 5 mM and 10 mM $MnSO_4$ alone to the germination media had little observable effect on the levels of shoot emergence (Fig. 3.45), and a small inhibitory effect on germination levels at day one of germination only (Fig. 3.44). Still, the addition of the three $MnSO_4$ concentrations were observed as having a concentration dependent inhibitory effect on average rootlet length measurements from approximately day three of germination possibly showing that later stages of rootlet growth and development are sensitive to external manganese levels.

The effects of the addition of 5 mM $MnSO_4$ and different concentrations of $ZnSO_4$ on the 5 mM 1, 10 phenanthroline mediated inhibition of protease activity during germination was also investigated (Figs. 3.42 and 3.43). In these studies both $MnSO_4$ and $ZnSO_4$ inclusion failed to rescue protease activity levels to those of the control grains showing that although both

$MnSO_4$ and $ZnSO_4$ can rescue germination they may not be as important for protease activity, indicating that maybe some but definitely not all of the proteases active during barley grain germination relay on manganese or zinc as either a catalytic or activating co – factor (Hänsch & Mendel, 2009) for their activity. Furthermore, since the 1, 10 phenanthroline mediated inhibition of germination was rescued by the inclusion of manganese and zinc in the germination media, these studies also show that germination is not dependent upon high levels of metalloprotease activity.

Together these observations illustrate the levels of complexity in the involvement of the different transition metal ions in the processes of barley grain germination as all three transition metals investigated here were shown to be important for barley grain germination levels, but were shown to have varying levels of effectiveness in the growth and developmental processes which accompany germination. There is thus a large body of future work that could follow from these studies to further elucidate the exact roles and influences that these metals have in barley grain germination. This work could involve investigations into their influence on plant growth hormones such as GA or ethylene (as both iron and manganese are known to be involved in GA biosynthesis (Hänsch & Mendel, 2009)), how the transition metals are utilised by the grain during the germination process and also genetic differences for the effects of these metal on germinating barley grains.

As previously mentioned, the non – transition metal divalent cations investigated (magnesium and calcium) had little effect on the 1, 10 phenanthroline mediated inhibition of germination (Figs. 3.33 to 3.41). However, when the effects of the addition of 1 mM, 5 mM and 10 mM $CaCl_2$ alone on the above processes were examined (Figs. 3.36 to 3.38) it was observed that at days one to two of germination the addition of $CaCl_2$ produced a decrease in the germination levels of barley grains, and also (on days three to five) a decrease in the levels of both shoot emergence and rootlet length indicating that while exogenous calcium may have little role in these processes (as addition of the three $CaCl_2$ concentrations failed to effect the 1, 10 phenanthroline mediated inhibition) the grains are still sensitive to elevated levels of calcium.

4.4: Metalloprotease and Serine Protease Enrichments

The goals of the metalloprotease and serine protease enrichment experiments were to attempt to identify and characterise members of these two protease classes within barley grain extracts. Despite the different extraction techniques employed and the different starting materials (four day kilned malt for the metalloprotease and ungerminated barley grains for the serine protease enrichment) due to the large number of proteins still present in the FPLC fractions at the last stage of each enrichment procedure, neither method resulted in SDS – PAGE gels with few enough bands for positive protease identification by Maldi – ToF mass spectroscopy. This issue of high numbers of interfering proteins present within the FPLC fractions was addressed by a number of "cleaning up" steps of the initial sample for the metalloprotease enrichment including the use of ammonium sulphate precipitation as a method of removing unwanted proteins. However, due to the ability of proteases to self – digest, they are inherently unstable. Throughout the metalloprotease and serine protease enrichment procedures the issue of protease stability was a recurrent theme as for example, throughout both the fractionation process and the ammonium sulphate precipitation step for the metalloprotease enrichment, the levels of protease activity fell and even disappeared completely. Overcoming these protease instability issues was attempted during the serine protease enrichment process by the addition of an alternate substrate (BSA) into the extract prior to loading onto the FPLC. Furthermore, all extracts and fractions were kept on ice (to lower the levels of protease activity, this was also carried out during the metalloprotease enrichment process) throughout and inhibitors of other protease classes were added (E – 64 and EDTA). In addition, in order to obtain a cleaner starting sample, an alternative extraction procedure was employed using "cracked" ungerminated barley grains and germinating them in a sodium succinate based extraction buffer, in the presence of GA_3, and changing the buffer every 24 hours. This was modified from a procedure that was successfully used to identify the cysteine protease EP-A (Koehler & Ho, 1988). This protocol did result in fewer bands being observed on SDS – PAGE gels but there were still too many proteins for positive protease identification by Maldi – ToF mass spectroscopy.

In 2008 Schmitt & Marinac demonstrated, using 2D zymograms, that there were several protease activities present in green malt crude extracts that could degrade barley grain β – amylase. Furthermore, they showed that these protease activities could be inhibited by the class specific serine protease inhibitor PMSF leading them to postulate that serine proteases could be involved in the degradation of β – amylase during barley grain germination. With this in mind the serine protease enrichment was aimed at the purification of one or more of

these potential serine protease activities and, despite not being able to purify a serine protease, this investigation did provided further evidence for the involvement of the serine class proteases in the degradation of β – amylase as day three extract (the extract day which exhibited the highest levels of serine protease activity (Fig. 3.60)) was shown to inhibit the activity levels of pure β – amylase, and that the more day three extract added the greater the inhibition of β – amylase activity (Fig. 3.61). Due to stability issues with the protease activities from FPLC fractionation no further investigations were able to be carried out into the effects of the addition of samples from the serine protease activity containing fractions from the FPLC on the activity of the pure β – amylase.

4.5: Starch Degrading Enzymes

In order to further investigate the roles of the four protease classes in overall grain physiology the effects of the different class specific protease inhibitors on the activities of three major starch degrading enzymes (limit dextrinase, β – amylase and α – amylase) were investigated. These three enzymes were chosen as they are not only important to grain germination, but are also principal contributors to the diastatic potential of barley malts (Gibson et al, 1995). The diastatic potential of malts is a very important parameter in defining malt quality as it represents the sum total of the activities of a number of starch degrading enzymes including limit dextrinase, α – amylase and β – amylase. Therefore, determining the influence of barley grain proteases on the activities of these enzymes could have important consequences for future malt quality parameters.

4.5.1: Limit Dextrinase

The effects of the four class specific protease inhibitors on the activities of limit dextrinase were investigated by adding them to the extraction stage and (in separate assays) the assay stage of commercial limit dextrinase assay kits using four day kilned malt (Fig. 3.47). No assays showed any change in limit dextrinase activity when the inhibitors were added at the assay stage. However, when the inhibitors were added at the extraction stage, that is the point at which limit dextrinase is being released from its inhibitor proteins, a reduction in limit dextrinase activity was seen in the presence of the class specific cysteine protease inhibitor E – 64 and also, to a lesser extent, in the presence of the class specific metalloprotease inhibitor 1, 10 phenanthroline. These results showed that cysteine protease activity and also possibly metalloprotease activity, or the presence of divalent cations, are required for limit

dextrinase activation; this is a result that is supported by the literature which indicates that cysteine protease activity is required for the release (and thus activation) of limit dextrinase from its inhibitor proteins (Longstaff & Bryce, 1993). Additionally, when the reducing agent DTT was omitted from the extraction stage a large decrease in the activity of limit dextrinase was observed (Fig. 3.47). This result is also consistent with the literature as reducing conditions are known to be very important for limit dextrinase activation, most probably through reduction and thus activation of cysteine proteases (Longstaff & Bryce, 1993, Heisner & Bamforth, 2008). The inhibition of limit dextrinase activity when 1, 10 phenanthroline was added to the extraction buffer could be the first evidence of a role for the metalloprotease class in the activation of limit dextrinase. However, since reducing conditions are also required for limit dextrinase activation, it could be that the chelation of transition metal divalent cations (such as Fe^{2+} for example (Palmer & Guerinot, 2009)) by 1, 10 phenanthroline could result in an alteration of the redox state of the assay thus inactivating the cysteine proteases responsible for limit dextrinase activation.

4.5.2: β – Amylase

β – amylase is one of the most important components of the diastatic power of malt as there is very close correlation between the levels of β – amylase activity in a malt and its diastatic power (Gibson et al, 1995 and Georg – Kraemer et al, 2001) thus a full knowledge of the physiological activation and activity of β – amylase would be very useful in developing new malting varieties with improved diastatic properties. Preliminary β – amylase activity studies were carried out on four day kilned malt using a commercially acquired β – amylase assay kit. The class specific protease inhibitors were added at the extraction stage of the assay and, in a different set of assays, at the assay stage (Figs. 3.54 and 3.55 respectively). The only inhibitor of the four tested to produce an appreciable decrease in β – amylase activity when added at the extraction stage was the class specific aspartate protease inhibitor pepstatin – A (Fig. 3.54). This decrease in β – amylase activity was also observed during germination studies where germinating barley grains in the presence of pepstatin – A produced a significant ($p = < 0.05$) decrease in total β – amylase activity on days two and three of germination. These results could be interpreted as the aspartate class proteases having a role in the release of bound β – amylase from its inhibitors or in the positive regulation of the amounts of β – amylase present in the grains during germination. However, if aspartate class proteases were involved in the release of bound β – amylase from its inhibitors it would be expected to see a significant decrease in the levels of free (i.e. unbound) β – amylase also. However, when the levels of free β – amylase activity were

measured (by omitting the reducing agent cysteine from the extraction buffer) no significant change in β – amylase activity was observed in the presence of pepstatin – A (Fig. 3.58) indicating that the aspartate class proteases are not involved in the release of bound β – amylase or in the positive regulation free β – amylase protein amounts. This lack of aspartate class protease involvement in the regulation of β – amylase activity is supported by a study in which the release of bound β – amylase extracted from barley grains was investigated by incubating the bound β – amylase with extracts from endosperms excised from germinating barley grains, and also with class specific protease inhibitors (Sopanen & Lauriere, 1989). This study showed that pepstatin – A had no inhibitory effect on the release and thus activity of the bound β – amylase. On the other hand, it is known that free β – amylase is easier to extract than the bound form (Evans et al, 1997) so it could be possible that the differences observed between the four day malt, total β – amylase and the free β – amylase could be a result of this difference in extractabilities of the β – amylase forms. Further studies using western blot analysis or ELISA assays (Evans et al, 1997) of the amounts of β – amylase proteins present with pepstatin – A , and also further studies into the β – amylase inhibitors and the process of β – amylase release from these proteins may shed more light on the possible reasons for the differing results of the current study.

When the different class specific protease inhibitors were added at the assay stage of the four day malt assays a reduction in the levels of β – amylase activity was observed in the presence of the divalent cation chelator and metalloprotease inhibitor, 1, 10 phenanthroline (Fig. 3.55). Since all of the β – amylase should have been released during the extraction stage of the assay, the inhibition of β – amylase activity observed in the presence of 1, 10 phenanthroline is most likely to be a result of the involvement of divalent cations in the activity of β - amylase rather than any role for the metalloproteases. The lack of a role for the metalloproteases in the release of bound β – amylase is supported by Sopanen & Lauriere, 1989, who showed that the addition of EDTA (a metalloprotease inhibitor) to assays with bound β – amylase (extracted from barley grains) and excised germinating barley grain endosperms had no inhibitory effect on the levels of β – amylase activity, indicating that metalloproteases, and also divalent cations, may not have a role in the release or activity of β – amylase during barley grain germination. However, 1, 10 phenanthroline was used instead of EDTA in this study because 1, 10 phenanthroline is more specific for zinc making it a better metalloprotease inhibitor than EDTA as, in general, the majority of metalloproteases are zinc dependent (Beynon & Bond, 2001).

However, a role for divalent cations in the activity of β – amylase was supported by assays using pure β – amylase (Fig. 3.56) in the present study. These studies showed that when pure β – amylase was assayed in the presence of both 5 mM 1, 10 phenanthroline and 20 mM $ZnSO_4$ the levels of β – amylase activity were partially rescued indicating that like α – amylase (which has a requirement for Ca^{2+} ions for its activity (Bush et al, 1989)), β – amylase requires the presence of divalent cations for its activity. The inhibitory effects of 1, 10 phenanthroline were also seen in the germination studies in both free and total β – amylase activity assays where 1, 10 phenanthroline significantly ($p = < 0.05$) inhibited β – amylase activity on days two to four and days two and five respectively (Figs. 3.57 and 3.58). This inhibition could be explained not only by the potential requirement of β – amylase for divalent cations such as zinc, but also by the inhibition of germination that 1, 10 phenanthroline produces, as β – amylase releasing factors could all be produced *de novo* or activated during the germination process. Unexpectedly, there was an increase in the levels of total β – amylase activity in the presence of all inhibitors tested, including 1, 10 phenanthroline, at germination day four (Fig. 3.57). This could be due to the unusually low control values for this day of germination. The possible reasons for these lower than expected control values could be due to an issue in experimental technique (such as pipetting errors) in the total β – amylase activity assays on this day of germination. Thus, this experiment would have to be repeated in order to rule out experimental error.

The role of cysteine class proteases in the activation of β – amylase is well established in the literature (Sopanen & Lauriere, 1989, Grime & Briggs, 1996) thus it was surprising that the grains germinated in the presence of the class specific cysteine protease inhibitor E – 64 did not have any significant affects in the levels of free or total β – amylase activity (Figs. 3.57 and 3.58), with the exception of day five total β – amylase activity, where those grains germinated in the presence of E – 64 showed a significantly lower level of β – amylase activity compared to the control, a result that was not seen in day five of the free β – amylase activity experiments (Fig. 3.58). A possible reason for this unexpected result could be that cysteine proteases protect the β – amylase / inhibitor complexes from degradation by other protease classes during later stages of germination, however, further work would be required to determine whether this was the case or not.

The class specific serine protease inhibitor PMSF was shown to have little effect on the β – amylase activities the four day malt studies (Figs. 3.54 and 3.55), but was shown to have a

significant ($p = < 0.05$) stimulatory effect on the activity of both total and free β – amylase (Figs. 3.57 and 3.58) during the germination experiments on days one, four and five, and days two, three and four respectively. This increase in the activity of β – amylase fits well with the data from the serine protease enrichment studies (section 4.4) where the addition of varying volumes of day three germination extract (the extract day that was found to contain the highest levels of serine protease activity (Fig. 3.61)) resulted in a volume dependent decrease in the activity of pure β – amylase. Moreover, these results also support the data from Schmitt and Marinac (2008) that β – amylase is degraded during germination by serine class proteases.

4.5.3: α – Amylase

α – amylase is important during grain germination as it is one of the only known enzymes to be present in germinating barley grains that can initiate native starch hydrolysis (Georg – Kraemer et al, 2001). α – amylase is synthesised in an active form during grain germination in response to GA signalling, but due to the presence of α – amylase inhibitors within the grain, α - amylase inhibitor degradation is required for full α – amylase activity.

In preliminary investigations carried out using extracts from four day micro malted malt none of the four class specific protease inhibitors had any significant effect on the levels of α – amylase activity (Fig. 3.48), but when barley grains were germinated in the presence of the different class specific protease inhibitors a different picture emerged (Fig. 3.49) with all four class specific protease inhibitors having a significant inhibitory effect on one or more days of germination. The greatest amount of α – amylase activity inhibition occurred in the presence of PMSF at day two of germination (Fig. 3.49). This points to a potential role for the serine class protease in the positive regulation of α – amylase activity during germination either by the degradation of α – amylase inhibitors or by the positive regulation of the amounts of α – amylase protein present within the grains. A similar result was obtained from the germination of barley grains in the presence of the class specific aspartic protease inhibitor pepstatin – A (Fig. 3.49). In these grains there was a significant inhibition of α – amylase activity on days three to five of germination, also indicating a role for the aspartic class proteases in either α – amylase inhibitor breakdown or the positive regulation of the amounts of α – amylase protein present within the grains. With this in mind western blot analysis for α – amylase was carried out (Fig. 3.52) and showed that in the presence of PMSF and pepstatin – A there was a decrease in the amount of α – amylase protein present within the

grains at days three and four of germination. Furthermore, when grains were germinated in the presence of both PMSF and pepstatin – A together a larger reduction in α – amylase protein amount than was observed with either inhibitor alone, with very little α – amylase protein present until day four of germination (Fig. 3.52). This result was supported by assays of α – amylase activity carried out in the presence of PMSF and pepstatin – A together. These results collectively show that aspartate and serine class proteases are important for the amounts of α – amylase protein present within barley grains during germination and that the decrease in α – amylase activity observed in the presence of both PMSF and pepstatin – A is due to a reduction in the amounts of α – amylase protein and not just a reduction in α – amylase inhibitor degradation.

Since the aspartate class proteases are known to be present in the aleurone layer and endosperm of germinating barley grains (Zhang & Jones, 1995) and are proposed to have little importance for storage protein degradation (Jones, 2005) a role for members of this protease class in the positive regulation of α – amylase activity is a good explanation for both their localisation within the germinating barley grain and also their presence within the grain during germination. The serine class proteases are also not thought to be important for storage protein degradation as neither SEP – 1 nor Hordolisin have been shown to be active against purified hordeins (Jones, 2005). Also it has been postulated that the serine class proteases fulfil more regulatory roles during barley grain germination, a hypothesis supported by Schmitt & Marinac (2008) who found evidence that serine class proteases may be involved in the degradation of β – amylase during barley grain germination thus showing that serine proteases have been found to fulfil potential regulatory roles.

In this work both serine and aspartate class proteases were found to have potential regulatory roles in the promotion of the amounts of α – amylase present within the grain demonstrating the importance of these two protease classes during germination. It must be kept in mind however, that just because the serine and aspartate class proteases have been found to effect α – amylase protein levels it should not be assumed that the members of either both or one of these protease classes that are involved in this positive regulation of α – amylase protein levels are located in the endosperm during germination as it is unclear from the present study where about in the process of α – amylase expression these proteases have their involvement. Also, just because the serine class proteases have been shown here and in the study by Schmitt & Marinac (2008) to negatively regulate β – amylase

activity it does not necessarily follow that the same serine proteases are potentially involved in both processes as there are likely to be many individual protease of any particular class and each might have different and specific functions within the grain.

The serine and aspartate proteases were not the only protease classes to be shown to be potentially important for the activity of α – amylase during germination as inhibitors of metallo and cysteine class proteases were also shown to inhibit α – amylase activity during grain germination (Fig. 3.49). The class specific cysteine protease inhibitor, E – 64, was shown to differ in its effects compared to the other three class specific protease inhibitors investigated, as it only significantly inhibited α – amylase activity on days two and three of germination (Fig. 3.49) compared to days two to five for the other three class specific protease inhibitors. This shows that there could also be temporal differences in the regulation of α – amylase by the different protease classes and that they may have diverse roles in the regulation of α – amylase expression within the grains. For example, it could be possible that the cysteine class proteases only have a role in the early onset of α – amylase expression. However, further work is required to completely reveal the roles of these protease classes.

The class specific metalloprotease inhibitor, 1, 10 phenanthroline, also inhibited α – amylase activity and, like pepstatin – A and PMSF, it inhibited on days two to five of germination (Fig. 46). This result could show that metalloproteases are also important for the positive regulation of α – amylase activity during barley grain germination. However, unlike the other class specific protease inhibitors, 1, 10 phenanthroline exerts its inhibitory effects through the chelation of divalent cations such as Ca^{2+} which is known to be necessary for the synthesis, transport (Bush et al, 1986), stability and activity of α – amylase during grain germination (Bush et al, 1989). Thus it is most likely that the inhibition of α – amylase activity observed in the presence of 1, 10 phenanthroline is a reflection of this requirement of α – amylase activity for Ca^{2+} ions. Moreover, grains germinated in the presence of 1, 10 phenanthroline show inhibited levels of germination, and since α – amylase is synthesised during grain germination this is a probable reason for the lower levels of α – amylase activity observed in the presence of this inhibitor. It is known that some α – amylase activity is present in mature, resting grains and that this activity is produced by members of the high pI α – amylases (Georg – Kraemer et al, 2001) which are also synthesised *de novo* during early germination (Muralikrishna & Nirmala, 2005). Unlike their low pI counterparts, the high pI isoforms are calcium – independent and have been postulated to account for

approximately 40 % of the grains total α – amylase activity during germination (Muralikrishna & Nirmala, 2005), thus even though 1, 10 phenanthroline inhibits barley grain germination and may therefore inhibit α – amylase synthesis and the activity of the low pI calcium – dependent α – amylase isoforms, the α – amylase activity that was detected in the 1, 10 phenanthroline germinated grains could possibly be the result of the activity of the high pI α – amylase isoforms, however, since no day zero α – amylase activity measurements were made this hypothesis cannot be substantiated in this study.

To further investigate the roles of serine and aspartate proteases in the positive regulation of α – amylase during germination, investigations were carried out where pepstatin – A and PMSF were added to barley grains at different days of germination (Figs. 3.51 and 3.50 respectively) and the grains assayed for α – amylase activity on day five of germination. These investigations revealed that adding PMSF from the very start of the experiment (i.e. from day zero) had the greatest inhibitory effect on α – amylase activity indicating that the serine proteases are more important for α – amylase protein levels during the early stages of germination. This is further evidenced by the lack of any significant inhibition of α – amylase activity when PMSF was added on day three of germination. But in the grains in which PMSF was added during day four of germination there was also a significant reduction in the levels of α – amylase activity showing that although serine proteases may be more important for the levels of α – amylase activity during early germination, they may also have a smaller involvement during later stages (e.g. in the activation of certain members of the low pI α – amylase isoforms). Part of the role for the serine proteases in the positive regulation of α – amylase may reside in the 26S proteasome, as in order for GA responsive genes, such as the α – genes to be induced, DELLA proteins (negative regulators of GA signalling (Ueguchi – Tanaka et al, 2005)) need to be degraded (section 1.2.5). This degradation is carried out by their ubiquitination and then degradation by the 26S proteasome (Wang & Deng, 2011). The 26S proteasome is known to contain trypsin – like and chymotrypsin – like protease activities (Sullivan et al, 2003), thus it could be that part of the role of the serine proteases in the positive regulation of α – amylase protein levels during germination is due to their involvement in the 26S proteasome and the degradation of DELLA proteins. It should be noted, that to avoid dehydration stress more water (and inhibitor) was added after three days of germination to the day zero and day one grains, thus the inhibitory effects of PMSF (and also pepstatin – A) in these grains should reflect this double PMSF dose. However, PMSF has a short half life in aqueous solution (just under two hours) (James, 1978) so PMSF added at day zero would have had its effects in a very short time frame, probably limited to

the start of germination, thus the large and significant decrease in α – amylase activity observed in these grains would be expected to be largely due to the importance of serine protease activity during early germination, rather than any major influence of the inhibition of serine proteases by the extra dose of PMSF at day three of germination. This hypothesis could be investigated by carrying out the germination experiments again, but this time not adding any PMSF along with the day three extra dose of water.

The pepstatin – A results revealed a slightly different picture (Fig. 3.51) as the addition of pepstatin – A at day four of germination produced the largest decrease in α – amylase activity, furthermore there was a significant reduction in the levels of α – amylase activity with addition at each day of germination. This could show that unlike the serine proteases, aspartate proteases are equally important throughout the germination process with the possible exception of later germination where they may become more important than the serine proteases for the amounts of α – amylase present, possibly through the positive regulation of the low pI α – amylase isoforms which are active during the later stages of germination (Muralikrishna & Nirmala, 2005).

Western blot analysis of PMSF and pepstatin – A germinated grains showed almost no α – amylase protein at day two in the control, PMSF and pepstatin – A germinated grains, and almost none in the day two and three pepstatin – A plus PMSF grains, but the assays showed some level of α – amylase activity at these times under all inhibitor and control conditions, apart from PMSF at day two of germination in which no α – amylase activity was observed. Since the antibody used was specific for all α – amylase isoforms the reason for the differences observed between the assays and western blot could be that the western blot was not sensitive enough to pick up small amounts of α – amylase present within the grain. Further studies are therefore required to obtain a more representative overview of the temporal importance of these proteases in α – amylase activity.

4.5.4: α – Amylase and Gibberellic Acid

α – amylase is synthesised in the barley grain aleurone layer in response to GA signalling (Varner, 1964). To investigate whether the reduction in α – amylase protein levels observed in the presence of the class specific protease inhibitors PMSF and pepstatin – A were due to the involvement of the serine and aspartic class proteases in this GA signalling process a

further set of germination studies were carried out (Fig. 3.53). These studies showed that when grains were germinated in the presence of varying concentrations of GA_3 alone there was, as expected, a significant concentration dependent increase in the levels of α – amylase activity during all three germination days investigated (Fig. 3.53). When the barley grains were germinated in the presence of PMSF and / or pepstatin – A with varying GA_3 concentrations, there was also a GA_3 concentration dependent increase in the levels of α – amylase activity but not as high as the levels observed when GA_3 was added alone, indicating that the serine and aspartate proteases may not only have a role in the perception of the GA signal by the aleurone layer, but that they could also have a role in events upstream of GA perception, as if their roles were confined to GA perception it would be likely that no increase in α – amylase activity would be observed in the inhibitor plus GA_3 grains as the signal perception pathway would still be blocked by the effects of PMSF and pepstatin – A. Thus these results indicate potential roles for the serine and aspartate class proteases in both GA signal perception at the aleurone, and in the processes upstream of this such as GA post transcriptional processing or transport to the aleurone for example.

When the pepstatin – A and PMSF results were compared (Fig. 3.53) the levels of α – amylase activity were always higher on all days and for all GA_3 concentrations (apart from the 50 μM GA_3 addition on day two) in the presence of pepstatin – A than those observed when GA_3 was added along with PMSF. These observations can be interpreted as showing that the aspartate proteases have less of a role in the GA signalling pathway or that the aspartate class proteases are more sensitive to changes in the levels of GA than their serine protease counter parts. The former hypothesis is supported by the observations that when pepstatin – A and PMSF were added individually in the absence of GA_3 (Fig. 3.49), pepstatin – A produced no significant decrease in α – amylase activity until day three of germination, whereas PMSF induced total inhibition on day two and produced significantly lower α – amylase activity levels than either the pepstatin – A or control grains on days four and five of germination. However, western blot analysis of the levels of α – amylase protein present in PMSF and pepstatin – A germinated barley grains appeared to show that those grains germinated in the presence of pepstatin – A had less α – amylase protein than the PMSF grains (Fig. 3.52), thus it could also be possible that the aspartate proteases could have a greater role in the positive regulation of α – amylase protein levels than the serine proteases but that the serine proteases may be more involved in the degradation of α – amylase inhibitors thus despite there being more α – amylase protein present in the PMSF

grains than in the pepstatin – A grains, there is less α – amylase activity due to decreased levels of inhibitor degradation.

The results of the investigations into the effects of class specific protease inhibitors on the activity of α – amylase during barley grain germination have shown that the aspartate and serine protease classes are positive regulators of the amounts of α – amylase protein present in the grains in a process which may be involved in the upstream events of gibberellic acid signalling. However, these results leave a lot of questions still to be answered, such as whether the serine and aspartate proteases effect all the α – amylase isoforms as even though the high pI isoforms are already present in the mature grain it does not necessarily mean that they are immune from regulation as α – amylase inhibitors are also present in the mature grain. Also the question of where in the events leading up to GA signal perception the aspartate and serine proteases are involved needs to be answered along with the identification, purification and characterisation of the individual serine and aspartate proteases involved. Further to this, studies arising from the data here that serine and aspartate proteases are involved in the positive regulation of α – amylase protein levels and that serine proteases maybe involved in the negative regulation of β – amylase (a result supported by Schmidt and Marinac, 2008) could lead to investigations into the effects that PMSF, pepstatin – A , and also other class specific protease inhibitors, may have on malt quality and plant viability as both α and β – amylase are major constituents of the diastatic potential of malt and are also major players in the supply of energy (through starch breakdown) to the developing embryo. Thus changes in the activities / amounts of these two enzymes in the malting / germinating barley grain could have implications on malt quality and embryonic and seedling growth and development and could thus provide further information on the potential roles of these and other protease classes in overall grain physiology. Furthermore, just because these two protease classes have been shown to effect amylases it does not necessarily follow that members of these classes do not have other roles within the grain, thus investigating the effects of these and other class specific protease inhibitors on malt quality and plant viability could shed light on further roles of the proteases in malting and germination.

4.6: Conclusions

This study has shown that the scope of activity of the protease enzymes of germinating, and thus malting barley grains, is not limited only to the mobilisation of the grain's protein stores,

but encompasses the regulation of starch degrading enzymes and thus the mobilisation of the grains carbohydrate reserves. Furthermore, the observations that the serine class proteases positively regulate the levels of α – amylase present in grains during germination and also have a potential role in the negative regulation of β – amylase activity points to the complexity of the regulatory processes occurring during grain germination and malting, and also how finely balanced and regulated these processes need to be within the grain.

What's more, the present study has also shown that all four protease classes have similar pH optima indicating that the different protease classes may either be active in the same or similar grain or cellular compartments (within the endosperm for example) with the exception of the metalloproteases which may have additional activities in specific regions within specific grain compartments. For example the metalloproteases could be active within a vacuole within the embryo or aluerone layer thus separating them from the cellular conditions experienced by other proteases present within the same grain compartment. These results thus show that there could spatial as well as temporal differences in the activities of the different protease classes during barley grain malting and germination.

It has also been shown that the divalent cation chelator and class specific metalloprotease inhibitor 1, 10 phenanthroline inhibits barley grain germination and that this inhibition can be overcome by the addition of equimolar concentrations of transition metal divalent cations serving to highlight the importance of these ions in the process of barley grain germination. Furthermore, the observations made on the effects of 1, 10 phenanthroline and representatives of the transition metal ions on different aspects of barley grain germination has brought to light potential differences in the importance and possibly temporal involvement of the different transition metals investigated in the processes of barley grain germination.

This study has provided new and further information on the roles of the different protease classes in malting and germination but, due to the limited understanding of the identities of individual proteases and the difficulties faced in this investigation with their purification there is a need to extend this work to further elucidate not only the identities of the barley grain proteases but also their roles, and those of the transition metal ions in the complex and interweaving processes of barley grain malting and germination.

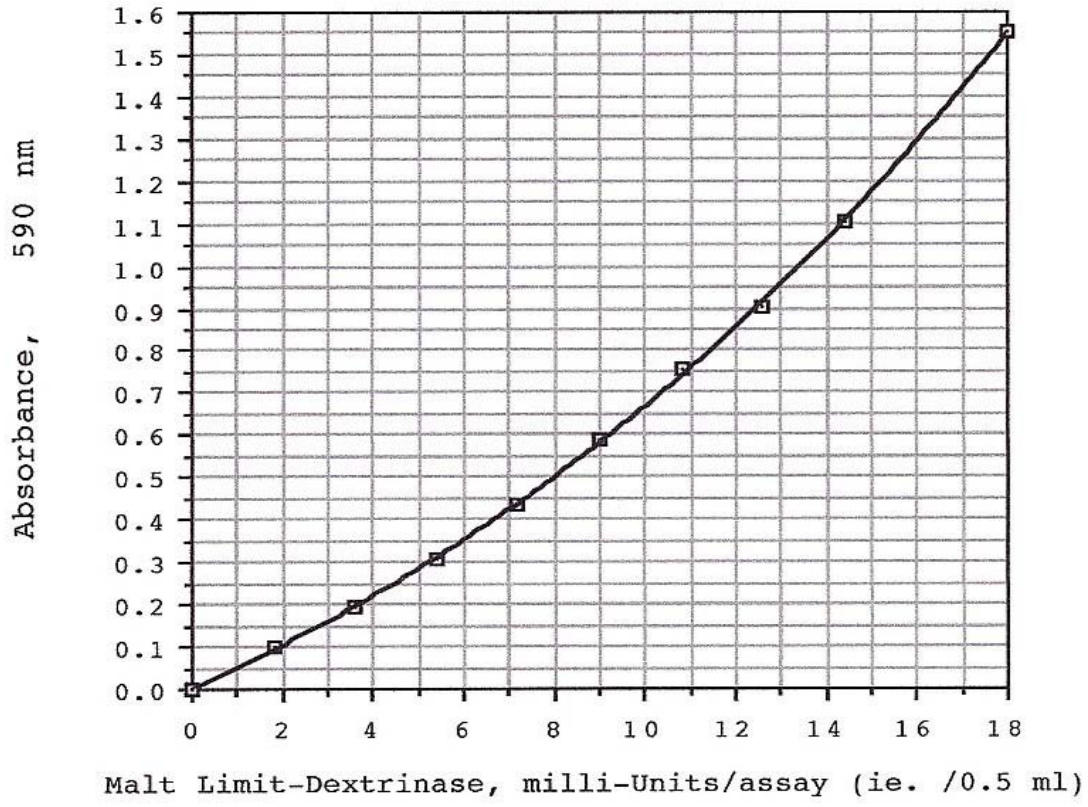


Figure 5.1: Limit – dextrizyme standard curve on Limit – Dextrizyme (adapted from "The Limit – Dextrizyme Method", Megazyme International Limited, Ireland)

Units of activity were calculated as follows:

The limit – dextrinase activity was determined by reference to the standard curve in Fig. 5.1. The standard curve converts the UV absorbance values into milliUnits of limit – dextrinase activity per assay:

Units of limit dextrinase activity / Kg Flour
= milliUnits per assay (that is per 500 µl) x (1 / 1000) x 32000
= milliUnits per assay x 32

Where:

1 / 1000 = converts milliUnits to Units; 32000 is the conversion factor used to change the activity / sample to the activity in 1 Kg of flour as the flour is extracted in 16 ml of extraction buffer per g flour, and the assay is carried out on 500 µl of extract, thus (16 x 1000) / 0.5 = 32000

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